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Specific, Active, Intradermal Immunization against Leukemia in Guinea Pigs[1]

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It has long been observed that spontaneous regression of an implanted tumor is followed by the establishment of a solid, specific, active immunity directed against this tumor [5]. However, spontaneous regression of homologous tumors growing in susceptible animals is a rare phenomenon. In our early studies carried out with BESREDKA, we observed that small doses of tumor cells implanted intradermally may in some instances, with proper dosage and proper technique of intradermal inoculation, lead to the development of tumors which remain localized within the skin, grow only temporarily, and eventually regress spontaneously. Animals which recover from such tumors develop solid immunity and resist reinoculation with large doses of the same tumors by any route. These initial experiments were carried out with the Ehrlich sarcoma on mice [1, 2], with the Rous sarcoma on chickens [4], and with the Brown-Pearce epithelioma on rabbits [3]. More recently, a similar specific immunity was established in C3H mice, by the method of intradermal immunization against a sarcoma that had been induced in these animals with methylcholantrene [7, 8].

Attempt to Induce a Specific, Active Immunity against Leukemia in Guinea Pigs

In recent experiments carried out in our laboratory [9] we have been transmitting, by serial subcutaneous cell graft passage, the L2C strain of guinea pig leukemia. This strain of leukemia originated as

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spontaneous leukemia in one of 'strain 2' guinea pigs bred and maintained at the National Cancer Institute. It was originally described by CONGDON and LORENZ [6] and has been carried since 1954 by serial cell graft transfer in 'strain 2' guinea pigs [10, 11]. We have confirmed in recent studies that this strain of stem-cell leukemia is uniformly leukemogenic, on cell graft transfer, for 'strain 2' or F_1 hybrid guinea pigs, and that it induces in these animals a rapidly progressive and uniformly fatal stem cell leukemia [9].

In the course of these experiments it occurred to us that it would be of interest to determine whether inoculation of small doses of leukemic cell suspensions directly into the skin of susceptible guinea pigs may not lead to the development of small intradermal tumors which would, in some instances at least, eventually regress, leaving these animals immune to reinoculation of heavy doses of leukemic cells. This experimental plan was based on our previous experiments carried out initially on mice [1, 2].

Leukemic L2C strain and 'strain 2' guinea pigs. The L2C leukemic strain, originally obtained from Dr. JUNGEBLUT from Lenox Hill Hospital, New York City, and carried in our laboratory by serial cell passage [9], has been employed in this study.

'Strain 2' guinea pigs, bred by brother-to-sister mating in our laboratory and in some instances also obtained from Horton's Laboratory Animals Inc. in Los Gatos, Calif., were employed in this study. In a few instances F_1 hybrids born in our laboratory to Hartley females and 'strain 2' males were also used.

Preparation of leukemic cell suspensions. A guinea pig with advanced leukemia was sacrificed by ether inhalation. After the skin was shaved, the abdominal cavity was exposed. Fragments of spleen, of the mesenteric tumor, and in some instances also of a peripheral lymph node were removed aseptically, weighed, cut with scissors, and ground in a mortar, sterile physiological saline solution being added to obtain a cell suspension of 10% concentration. The cell suspension was then passed through a sterile voile cloth and used immediately for inoculation.

Intradermal inoculation of small doses of leukemic cell suspensions. As a first step in our procedure, the smallest dose which consistently produced leukemic tumors following either intradermal or subcutaneous inoculation of leukemic cell suspensions was determined. We have reported previously [9], and confirmed in our current studies, that subcutaneous inoculations of 10^{-3} and 10^{-4} dilutions of leukemic cell suspensions into young adult 'strain 2' guinea pigs consistently induced progressively growing leukemic tumors leading to generalized stem-cell leukemia.

In an attempt to determine whether inoculation of similar doses of leukemic cell suspensions by intradermal route would have the same effect, a series of experiments was carried out in which small doses of leukemic cells were carefully inoculated directly into the skin of the right or left flank, on the back of the animal. Accordingly, in a small area on the flank of the guinea pigs, the hair was clipped closely with an electric clipper, the skin was lightly sponged with a 70 percent ethyl alcohol solution, and a small volume (0.1 to 0.2 ml) of a leukemic cell suspension of 10^{-3} to 10^{-6} concentration was then inoculated intradermally, using a 26 gauge needle. The skin of guinea pigs is rather thick, particularly in the males, the needle had to be introduced carefully so as to remain within the skin and not to penetrate into the subcutaneous tissue. The intradermal inoculation, if properly made, produced a small, elevated wheal at the site of inoculation.

Some intradermal tumors regressed spontaneously. In the first series 12 young adult 'strain 2' guinea pigs were inoculated with a 10^{-3} dilution of leukemic cell suspensions. Within 10 to 13 days, 9 of the 12 inoculated animals developed small intradermal tumors at the site of inoculation. Three guinea pigs did not react to the inoculation. In 2 out of 9 guinea pigs which developed intradermal tumors, the small intradermal tumor grew only temporarily, reaching about 3 mm in diameter, and then regressed without trace after 4 to 9 days.

In the remaining 7 guinea pigs, the intradermal tumors persisted and gradually enlarged, reaching eventually a diameter of 5 to 8 mm. At that point, metastatic tumors developed in the adjoining axillary lymph nodes and before long the disease became generalized. The peripheral blood morphology began its characteristic change at the time the metastatic tumors appeared and the peripheral white blood counts gradually increased to 100,000 and later exceeded 300,000 WBC/mm³, with large numbers of stem cells appearing in peripheral blood smears. All 7 animals died with generalized leukemia.

The intradermal tumors which later regressed, as well as those which grew progressively, were true leukemic tumors. Microscopic and electron microscopic studies of these tumors, still in progress, will be reported separately.

It was of considerable interest that those animals in which the intradermal tumors grew progressively, developed widespread disseminated metastatic tumors more pronounced and more striking

than the metastatic dissemination observed in those guinea pigs that had been inoculated routinely by the subcutaneous route A similar observation was made in our previous studies carried out on mice [1, 2]

In a second series, 10^{-4} dilutions of leukemic cell suspensions were inoculated intracutaneously (0 1 to 0 2 ml each) into 14 young adult 'strain 2' guinea pigs Three animals did *not react to the inoculation*, the remaining 11 guinea pigs developed small tumors at the site of inoculation after a latency of 10 to 20 days In 5 guinea pigs the intradermal tumors grew only temporarily, reaching about 3 mm in diameter and then gradually regressed without trace within approximately 4 to 10 days after their appearance In the remaining 6 animals the intradermal tumors increased in size gradually, during that time the peripheral blood counts in these animals remained normal Rather unexpectedly, however, metastatic tumors developed and became distinctly palpable in the adjoining axillary area, from then on, the disease became rapidly progressive, this was reflected in the gradually increasing number of white blood cells and the appearance of primitive white cells in peripheral blood In a few instances the increase in the white blood cell counts, and the appearance of abnormal white cells in the peripheral blood preceded by 2 or 3 days the development of palpable metastatic lymph nodes in the axillary area Within a short time, generalized leukemia and multiple metastatic leukemic tumors developed in these animals

In a third series, 10^{-5} and 10^{-6} dilutions of leukemic cell suspensions were inoculated intradermally into four 'strain 2' guinea pigs All 4 guinea pigs developed intradermal tumors at the site of inoculation after a latency of 12 to 22 days In 3 of them, the small intradermal tumors regressed spontaneously after 4 to 9 days (fig 1), and the *fourth animal died with generalized leukemia (table I)*

Intradermal inoculation of small doses of leukemic cell suspensions into F_1 hybrids In another similar experiment 10^{-5} dilutions of leukemic cell suspensions were inoculated intradermally into 3 F_1 hybrid guinea pigs and 10^{-4} dilutions were inoculated intradermally into 4 F_1 hybrids In the first group all 3 animals developed progressively growing tumors leading

In summary, in 13 out of 31 guinea pigs (42%), the intradermal tumors regressed spontaneously (table I)



a



b

Fig. 1. This 'strain 2 [illegible]
of a 10^4 dilution of le[illegible]
developed at the site o[illegible]
ed spontaneously wit[illegible] [illegible] the intradermal tumor a
the peak of its development. b 6 days later the intradermal tumor disappeared completely

Table I Results of intradermal inoculations of small doses of leukemic cell suspensions, compared with subcutaneous inoculations

Strain of guinea pigs inoc	Route of inoc [1]	Leuk cell conc	No guinea pigs inoc	No dev i d (or s c) tumors[2]	No i d (or s c) tumors regress	No dev gener leuk	No remain negat
'Strain 2'	i d	10^{-3}	12	9	2	7	3
F_1 hybrids	i d	10^{-3}	3	3	0	3	0
'Strain 2'	i d	10^{-4}	14	11	5	6	3
F_1 hybrids	i d	10^{-4}	4	4	3	1	0
'Strain 2'	i d	10^{-5}	2	2	1	1	0
'Strain 2'	i d	10^{-6}	2	2	2	0	0
Total	i d		37	31	13	18	6
'Strain 2'	s c	10^{-3}	10	10	0	10	0
F_1 hybrids	s c	10^{-3}	4	3	0	3	1
'Strain 2'	s c	10^{-4}	7	7	0	7	0
F_1 hybrids	s c	10^{-4}	3	3	0	3	0
'Strain 2'	s c	10^{-4}	3	3	0	3	0
Total	s c		27	26	0	26	1

[1] i d = intradermal (0 2 ml each), s c = subcutaneous (0 3 to 0 5 ml each)
[2] Average latency from inoculation to appearance of intradermal (i d) tumors, 12 days Those tumors that regressed spontaneously, disappeared after 2 to 10 days

Resistance of Animals in which the Intradermal Tumors Regressed, to Reinoculation of Leukemic Cells

We now had 10 'strain 2' and 3 F_1 hybrid guinea pigs, a total of 13 animals, in which the induced intradermal tumors had regressed spontaneously One of these animals is still under observation The remaining 12 were tested for their resistance to reinoculation of larger doses of leukemic cells As a preliminary procedure, 11 of them received additional intradermal booster doses of leukemic cell suspensions of 10^{-3} concentration, none reacted to the second intradermal inoculation The 12 guinea pigs were now challenged with subcutaneous inoculations, 0 5 ml each, of a 10^{-2} concentration of leukemic cell suspensions (table II) Among the challenged animals, the one that had not received the booster inoculation and one additional animal developed generalized leukemia The remaining 10

Table II Resistance of guinea pigs in which the intradermal tumors regressed spontaneously, to subcutaneous reinoculation of leukemic cell suspensions

Group of guinea pigs	Strain	No of guinea pigs challenged s c inoc [1]	No of guinea pigs develop leukemia	Average latency leuk dev days
Immunized [2]	'strain 2	9	2	26
	F_1 hybrids	3	0	
Total		12	2	
Controls	'strain 2	9	9	20
	F_1 hybrids	3	3	18
Total		12	12	

[1] 0.5 ml of 10^{-2} concentration of leukemic cell suspensions inoculated subcutaneously

[2] All guinea pigs in which the intradermal tumors regressed spontaneously, except one received a second intradermal inoculation (booster dose) 0 2 ml of 10^{-2} concentration of leukemic cell suspension prior to the challenging inoculation Of the 2 animals which did not resist the challenging subcutaneous inoculation, one had not received the booster dose

guinea pigs resisted the challenging inoculation and proved to be solidly immune They have been observed, thus far, for 3 to 5 months

In a control series, 9 normal young adult 'strain 2' guinea pigs and 3 F_1 hybrids were inoculated subcutaneously (0 5 ml each) with a 10^{-2} concentration of leukemic cell suspensions, and all 12 developed generalized stem cell leukemia within 14 to 20 days after inoculation After several weeks, an additional challenging inoculation was made on 4 of the immunized guinea pigs, 2 were reinoculated subcutaneously with 0 5 ml, each, of a heavy, 10% concentration of a leukemic cell suspension, and the other 2 were injected *intraperitoneally* with a 10^{-2} concentration of leukemic cells All 4 proved to be solidly immune and remained in good health In normal, untreated 'strain 2' or F_1 hybrid guinea pigs, such large doses of leukemic cells were consistently leukemogenic and induced a generalized, rapidly progressing disease within 20 days In an additional control experiment, one normal 'strain 2 and 2 normal F_1 hybrid guinea pigs were inoculated *intraperitoneally*, 0 5 ml each, with a 10^{-2} concentration of leukemic cells, and all 3 developed generalized leukemia after 10 to 14 days

Animals which Did not React to the Initial Intradermal Inoculation

The question remained to be answered whether those guinea pigs which did not react to the initial inoculation of leukemic cells would be resistant to a massive challenging reinoculation of leukemic cells, even though they had not initially developed, and recovered from, intradermal tumors.

The 6 guinea pigs which did not react to the initial intradermal inoculation (table I) were reinoculated intradermally with a 10^{-4} concentration of leukemic cell suspensions, and 3 of them developed progressively growing leukemic tumors leading to the development of generalized leukemia. The remaining 3 did not react to the reinoculation and remained in good health; they are still under observation.

It must be stressed, however, that observation and recognition of very small intradermal tumors which may develop following intradermal inoculations of small doses of leukemic cell suspensions *may be difficult*. Such intracutaneous tumors may be barely visible. We have noticed in a few instances that intradermal inoculation of guinea pigs with high dilutions of leukemic cell suspensions resulted, after a latency of 10 to 12 days, in the formation at the site of inoculation of an intradermal tumor so small as to represent only a slightly elevated, round thickening of the skin, about 2 or 3 mm in diameter. This very small growth disappeared without trace after 2 to 3 days. Such animals were then immune to reinoculation of leukemic cells. Accordingly, animals inoculated intradermally with small doses of leukemic cells should have their hair clipped closely at the site of inoculation, and they should be observed very carefully on a daily basis for 2 to 4 weeks in order to determine whether an intradermal tumor will not appear.

It is entirely possible that we could have missed a few of such small intradermal tumors in some of the animals observed, and that, consequently, among the guinea pigs that had been inoculated intradermally and were thought not to have reacted to the initial inoculation, and were therefore considered to be negative, there were some that actually did develop very small tumors which subsequently regressed, leaving such animals immune to a challenging reinoculation.

In our previous experiments carried out on mice, we have repeatedly made the observation that those animals which did not react to the initial inoculation of tumor cells and which did not develop tumors, were not rendered immune [1, 2, 7]. In those early studies we observed that only the development of a tumor, followed by spontaneous recovery, led to the development of specific immunity. However, the possibility must be considered that intradermal inoculations of very small doses of tumor cell suspensions, which may not necessarily lead to the development of a macroscopically recognizable tumor, may, nevertheless, result in the establishment of partial immunity which could later be reinforced by a subsequent intradermal inoculation of an additional 'booster' tumor dose. This possibility is now being investigated in our laboratory.

Results of subcutaneous inoculations. When, in contrast to intradermal inoculations, leukemic cell suspensions (0.3 to 0.5 ml, each) varying in concentration from 10^{-3} to 10^{-5}, were inoculated subcutaneously, practically all inoculated guinea pigs developed progressively growing leukemic tumors; these tumors developed at the site of inoculation after a latency varying from 10 to 25 days, depending on the con-

centration of leukemic cells employed for inoculation, however, all these tumors grew progressively and led to the ultimate development of generalized stem-cell leukemia, none regressed spontaneously (table I)

Only one F_1 hybrid guinea p g which received a subcutaneous inoculation of a 10^{-3} concentration of leukemic cells was found to be resistant, and did not react to the initial subcutaneous inoculation Subsequently this animal was reinoculated with additional doses of leukemic cell suspens ons but remained in good health

It appears from these studies that subcutaneous inoculation of leukemic cell suspensions into susceptible, young adult 'strain 2' or F_1 hybrid guinea pigs, consistently induces the development of generalized stem cell leukemia [2]

These experiments are now being continued small doses of h gh dilutions of leukemic cell suspensions are being inoculated subcutaneously in order to determine whether animals wh ch do not react initially to the inoculation of such small doses will be immune to reinoculation of a heavier dose of leukemic cells This was certainly not observed in our previous experiments carried out in mice [1 2 7] or chickens [4] and we doubt very much whether this would be the case in experiments on guinea p g leukemia However current experiments now carried out in our laboratory should give an answer to this question in the near future

Conclusions

It is apparent, therefore, that guinea pigs of a susceptible line could be immunized against inoculation of leukemic cells of L2C strain, which originated spontaneously in a guinea pig of the same inbred line, by a simple method consisting of inoculating intradermally a small dose of the leukemic cell suspension Among the induced intra dermal tumors, some regressed spontaneously The animals in which the tumors regressed were, with few exceptions, solidly immune to reinoculation of heavy doses of leukemic cell suspensions

These results are encouraging They demonstrate clearly that active, specific immunity can be induced in susceptible animals against experimental inoculation of leukemic cells

It remains to be seen whether those guinea pigs which recovered from the intradermal tumors will be also immune to virus induced leukemia, this point however, is difficult to determine at the present time, since, thus far at least, we have been unable to induce leukemia in guinea pigs with filtered, cell free, L2C leukemic extracts [9]

[2] For typ cal p ctures of stem-cell leukemia induced in 'strain 2 guinea pigs, the interested reader is referred to our preceding publication in Acta haemat. [9]

Summary

Intradermal inoculation of small doses of 10^{-3} to 10^{-4} dilutions of leukemic cell suspensions into 'strain 2' or F_1 hybrid guinea pigs produced intradermal leukemic tumors which regressed spontaneously in 13 out of 31 animals (42%). Twelve guinea pigs which recovered from the intradermal tumors were challenged, and 10 of them were found to be solidly immune to reinoculation of heavy doses of leukemic cells by either intradermal, subcutaneous, or intraperitoneal route. Inoculation of similar doses subcutaneously, or intraperitoneally, into 30 'strain 2' or F_1 hybrid guinea pigs induced generalized leukemia in 29 animals. None recovered from the induced leukemia.

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Catalase Activity of Immature and Mature Red Cells from Acatalasemic Mouse Mutant

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Acatalasemia was found first in Japan 1948 by TAKAHARA [1] and since then, it had been believed to be a molecular disease specific to some oriental races. In 1960, however, acatalasemia was also found in Switzerland [4]. The name of acatalasemia does not mean a completely lack of catalase activity in the blood, some catalase like activity or residual catalase activity has been demonstrated by manometric observation by TAKAHARA [2] in 1952 in Japanese and by AEBI [3-5] in 1962 in Swiss acatalasemia. The residual catalase activity in Swiss acatalasemia is kept mainly by the reticulocytes and their catalase is less resistant to heat than the catalase from normal individuals [6].

In 1964, FEINSTEIN [7-10] has succeeded in getting acatalasemic mouse mutant by irradiation, then catalase activity was 2.5% of normal level. This residual catalase activity was less resistant to heat than normal mouse catalase; it was presumed that the residual catalase should be different from normal catalase as to its molecular structure. The heat instability of the residual catalase of acatalasemic mouse also suggests that it is similar to that found in acatalasemic individuals in Swiss, and may be contained mainly in reticulocytes. From this view point, the authors aimed to observe catalase activity of reticulocytes of anemic acatalasemic mouse.

Materials and Methods

Thirty-two adult male mice about 25 g body weight were used. To induce hemolytic anemia, 7 normal (Cs^a) strains and 10 acatalasemic Cs^b mutants were injected with

neutralized phenylhydrazine chloride, 20 μg/g of body weight subcutaneously daily for 3 days [11, 12]. For blood depleted anemia, 5 acatalasemic (Cs^b), 2 homozygous hypocatalasemic (Cs^d) and 6 normal (Cs^a) and 2 heterozygous hypocatalasemic (Cs^{ab}) mice were bled from orbital vein, about 0.5 ml at one time and 8 times during 8 days. With appropriate intervals hemoglobin level, reticulocyte count and catalase activity were estimated on the blood taken from the orbital sinus [13]. Hemoglobin level was estimated by cyano-methemoglobin method by spectrophotometry. Reticulocyte counts were made on the smear of the blood which was preliminary stained with Nile-blue supravitally, fixed with methanol and stained with Giemsa [14]. The estimation of blood catalase activity was made by the method of Aebi [15], a modification of Feinstein's method [16] or by manometry [17]. The heat stability of catalase was observed on the hemolysed sample incubated at 44°C for 10 min in water bath.

Results

The repeated injections of phenylhydrazine hydrochloride induced a severe anemia, about 6 g/dl Hb concentration, with a marked reticulocytosis, 43–50%, both in normal and acatalasemic mice. The reticulocyte count reached the maximum 3 days after the final injection of the agent, then decreased. The catalase activity of the blood obtained 3 days after the final injection of phenylhydrazine showed an extremely high level as revealed by the perborate as well as manometric methods, and then decreased. The data clearly indicated that the catalase activity increased with development of anemic condition or with high reticulocytosis both in non-acatalasemic and acatalasemic animals. But in non-acatalasemic mice only a slightly increase of catalase activity was noted, while in acatalasemic mice, whose catalase activity were extremely low, the activity was markedly enhanced by reticulocytosis. However, the maximum level of the latter was still lower than that of the former (fig. 1).

In blood depletion the reticulocyte count increased with the repetition of blood drawing or the development of anemia and faded in the recovery stage of anemia in all cases. The catalase activity also increased with the increase in reticulocyte count, giving nearly the same results as in the phenylhydrazine anemia. The increasing rate of a catalase activity was the highest in acatalasemia, moderate in homozygous hypocatalasemia, and the lowest in normal and heterozygous hypocatalasemia.

The catalase activity of the whole blood increased in proportion with increase of reticulocyte count. From the regression equation showing the relationship between reticulocyte count and catalase

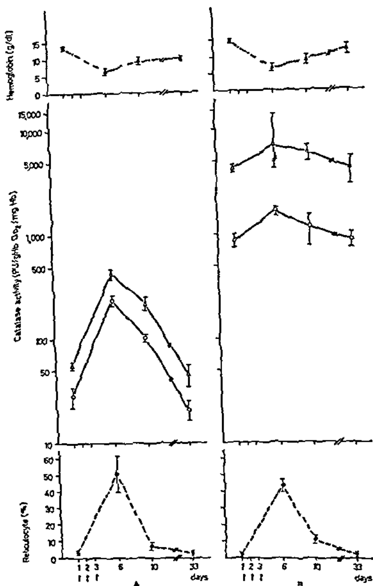


Fig 1 Changes in hemoglobin concentration, reticulocyte count and catalase activity in the bloods of acatalasemic Cs^b (A) and non-acatalasemic Cs^a (*B*) mice treated with phenyl hydrazine Each curve gives the mean value of 5 animals with standard error ▲ Hemoglobin concentration △ catalase activity by manometric method (Q_{O_2} mgHb), O catalase activity by perborate method (PU/gHb), ● reticulocytes. Arrows phenylhydrazine injection

activity, it is possible to calculate the catalase activity of reticulocytes and mature red blood cells.

In the phenylhydrazine anemia of non acatalasemic animal the catalase activity of the reticulocytes was 2622.0 PU/g Hb, and it decreased with the advance of the maturation reaching the value of normal mature red blood cells, 742.0 PU/g Hb. The decreasing rate of catalase activity of maturation of reticulocyte was found to be 0.72 in the non acatalasemic mice. The catalase activity of acatalasemic reticulocytes, 396.3 PU/g Hb, which was 15.1% of that of normal reticulocytes, decreased to 16.3 PU/g Hb by maturation to red blood cells. This value of acatalasemic mature red cells was only 2.2% of that of normal mature red blood cells. Thus in acatalasemic reticulocytes, the rate of decrease in catalase activity by maturation was 0.96, indicating a high degradation rate of catalase during the maturation (table I).

In the blood depleted anemia, the decrease in catalase activity by reticulocyte maturation occurred similarly as in the phenylhydrazine anemia in non acatalasemic and acatalasemic mice, though catalase activity of reticulocytes of acatalasemic mouse in blood depleted anemia appeared somewhat lower than that in phenylhydrazine anemia. It should be considered that, in phenylhydrazine anemia, almost all red blood cells are hemolyzed and relatively many younger reticulocytes are proved compared to mice of blood depleted anemia. A relative high catalase activity of acatalasemic mice in phenylhydrazine anemia may be due to the high activity of younger reticulocytes. Catalase activity of homozygous hypocatalasemic reticulocytes, 1763.6 PU/g Hb, which showed 66.9% of catalase activity of normal reticulocytes decreased by maturation to 183.6 PU/g Hb, which is 20.0% of catalase activity of normal mature red blood cells, and the rate of the decreased activity by maturation was 0.90. In heterozygous hypocatalasemic mice, the rate of degradation of catalase by maturation is calculated to be 0.62, and this value is similar to that of normal catalase, 0.65, and different from those of homozygous hypocatalasemic mice, 0.90 and acatalasemic mice, 0.92 (table I).

Degradation of the catalase by maturation of reticulocytes was also observed in the blood from the recovery stage of anemia, revealing the similar values to those found in the inducing stages of anemia (table I).

The ratio of catalase activity per cell of mature red blood cells to that of reticulocytes in non acatalasemic mice is examined to be 0.42

Table I Catalase activity of reticulocytes and mature red blood cells estimated in acatalasemic, homozygous and heterozygous hypocatalasemic, and normal mice in anemic condition

Method of inducing reticulocytosis		Mice	Reticulocytes	Mature red blood cells	Ratio of degradation[1]
Injection with phenylhydrazine hydrochloride		normal	2622.0 (100.0%)	742.0 (100.0%)	0.72
		acatalasemia	396.3 (15.1%)	16.3 (2.2%)	0.96
Bleeding	Inducing stage of anemia	normal	2636.3 (100.0%)	916.3 (100.0%)	0.65
		homozygous hypocatalasemia	1763.6 (66.9%)	183.6 (20.0%)	0.90
		acatalasemia	271.2 (10.3%)	21.2 (2.3%)	0.92
		heterozygous hypocytalasemia	957.5 (36.3%)	367.5 (40.1%)	0.62
	Recovery stage of anemia	normal	2700.6 (100.0%)	1100.6 (100.0%)	0.59
		homozygous hypocatalasemia	2079.0 (77.0%)	169.0 (15.4%)	0.92
		acatalasemia	260.5 (9.7%)	20.5 (1.9%)	0.92
		heterozygous hypocatalasemia	906.6 (36.5%)	526.6 (47.9%)	0.47

[1] Ratio of degradation = (activity in reticulocytes) − (activity in mature red blood cells) / activity in reticulocytes.

activity, it is possible to calculate the catalase activity of reticulocytes and mature red blood cells

In the phenylhydrazine anemia of non acatalasemic animal the catalase activity of the reticulocytes was 2622 0 PU/g Hb, and it decreased with the advance of the maturation reaching the value of normal mature red blood cells, 742 0 PU/g Hb The decreasing rate of catalase activity of maturation of reticulocyte was found to be 0 72 in the non acatalasemic mice The catalase activity of acatalasemic reticulocytes, 396 3 PU/g Hb, which was 15 1% of that of normal reticulocytes, decreased to 16 3 PU/g Hb by maturation to red blood cells This value of acatalasemic mature red cells was only 2 2% of that of normal mature red blood cells Thus in acatalasemic reticulocytes the rate of decrease in catalase activity by maturation was 0 96, indicating a high degradation rate of catalase during the maturation (table I)

In the blood depleted anemia, the decrease in catalase activity by reticulocyte maturation occurred similarly as in the phenylhydrazine anemia in non acatalasemic and acatalasemic mice, though catalase activity of reticulocytes of acatalasemic mouse in blood depleted anemia appeared somewhat lower than that in phenylhydrazine anemia It should be considered that, in phenylhydrazine anemia, almost all red blood cells are hemolyzed and relatively many younger reticulocytes are proved compared to mice of blood depleted anemia A *relative high catalase activity of acatalasemic mice in phenyl* hydrazine anemia may be due to the high activity of younger reticulocytes Catalase activity of homozygous hypocatalasemic reticulocytes, 1763 6 PU/g Hb, which showed 66 9% of catalase activity of normal reticulocytes, decreased by maturation to 183 6 PU/g Hb, which is 20 0% of catalase activity of normal mature red blood cells, and the rate of the decreased activity by maturation was 0 90 In heterozygous hypocatalasemic mice the rate of degradation of catalase by maturation is calculated to be 0 62, and this value is similar to that of normal catalase, 0 65, and different from those of homozygous hypocatalasemic mice, 0 90 and acatalasemic mice, 0 92 (table I)

Degradation of the catalase by maturation of reticulocytes was also observed in the blood from the recovery stage of anemia, revealing the similar values to those found in the inducing stages of anemia (table I)

The ratio of catalase activity per cell of mature red blood cells to that of reticulocytes in non acatalasemic mice is examined to be 0 42

Table 1 Catalase activity of reticulocytes and mature red blood cells estimated in acatalasemic, homozygous and heterozygous hypocatalasemic, and normal mice in anemic condition

Method of inducing reticulocytosis		Mice	Reticulocytes	Mature red blood cells	Ratio of degradation[1]
Injection with phenylhydrazine hydrochloride		normal	2622.0 (100.0%)	742.0 (100.0%)	0.72
		acatalasemia	396.3 (15.1%)	16.3 (2.2%)	0.96
Bleeding	Inducing stage of anemia	normal	2636.3 (100.0%)	916.3 (100.0%)	0.65
		homozygous hypocatalasemia	1763.6 (66.9%)	183.6 (20.0%)	0.90
		acatalasemia	271.2 (10.3%)	21.2 (2.3%)	0.92
		heterozygous hypocatalasemia	957.5 (36.3%)	367.5 (40.1%)	0.62
	Recovery stage of anemia	normal	2700.6 (100.0%)	1100.6 (100.0%)	0.59
		homozygous hypocatalasemia	2079.0 (77.0%)	169.0 (15.4%)	0.92
		acatalasemia	260.5 (9.7%)	20.5 (1.9%)	0.92
		heterozygous hypocatalasemia	986.6 (36.5%)	526.6 (47.9%)	0.47

[1] Ratio of degradation = (activity in reticulocytes) − (activity in mature red blood cells) / activity in reticulocytes

Table II Heat stability of catalase in acatalasemic, homozygous and heterozygous hypocatalasemic, and normal mouse reticulocytes

Method	Case	Normal mice		Acatalasemic mice		Homozygous hypocatalasemic mice		Heterozygous hypocatalasemic mice	
		Inactivating rate, %	Reticulocyte count, %	Inactivating rate, %	Reticulocyte count, %	Inactivating rate, %	Reticulocyte count, %	Inactivating rate, %	Reticulocyte count, %
Injection with phenylhydrazine hydrochloride	non treated	8.2	2.2	81.2	2.7	–	–	–	–
	3rd day after injection	8.9	43.3	87.5	51.7	–	–	–	–
Bleeding	non treated	9.9	1.9	85.2	1.5	87.6	2.7	35.6	2.0
	7th day after starting bleeding	11.3	48.9	93.1	47.7	91.7	39.3	47.3	53.4

Thus, the catalase activity per cell decreased by about 58% by the maturation of reticulocytes to the red cells In the case of the bloods of two acatalasemic mice examined, catalase activity per cell also decreased by about 96% by the maturation of reticulocytes

Heat stability of catalase in acatalasemic reticulocytes and mature red cells tested at 44°C was lower than that of non acatalasemic animal both in hemolytic and blood depleted anemia (table II) The data also indicate that the heat stability of catalase of reticulocytes of both phenylhydrazine anemia and blood depleted anemia was slightly less than that of mature red blood cells in both non acatalasemic and acatalasemic animals And also heat stability of acatalasemic reticulocytes in both phenylhydrazine and blood depleted anemias was less than that of normal reticulocytes Heat inactivating rate of catalase of heterozygous hypocatalasemic mice at 44°C for 10 min was 35 6%, and it was between that of normal catalase, 9 9%, and that of acatalasemic catalase, 85 2%

Discussion

Our studies revealed that the extremely low catalase activity of the mature red blood cells of acatalasemic mice was due to the low catalase activity, or minimized catalase synthesis, in reticulocytes and the considerably high degradation rate of the enzyme by maturation The homozygous hypocatalasemic mice, whose catalase activity of mature red cells was only about 2% of the non acatalasemic mice, showed a slight decrease in catalase activity of reticulocytes but the degradation rate of the enzyme by maturation was marked, showing nearly the same degradation rate as that of acatalasemic mice Therefore the lower activity of blood catalase in the hypocatalasemic mouse mutant is mainly due to the higher degradation rate of the enzyme by reticulocyte maturation and it is similar to that of acatalasemic individuals in Swiss, in which the lower catalase activity is mainly due to the decrease of catalase activity during the maturation of reticulocytes to red cells [6] The situation is completely different from the case of Japanese acatalasemic individuals [18–20] in which the catalase activity in reticulocytes is extremely low, about 0 2% of the non acatalasemic individuals but the activity does not decrease during the reticulocyte maturation

Table II Heat stability of catalase in acatalasemic, homozygous and heterozygous hypocatalasemic, and normal mouse reticulocytes

Method	Case	Normal mice		Acatalasemic mice		Homozygous hypocatalasemic mice		Heterozygous hypocatalasemic mice	
		Inactivating rate, %	Reticulocyte count, %	Inactivating rate, %	Reticulocyte count, %	Inactivating rate, %	Reticulocyte count, %	Inactivating rate, %	Reticulocyte count, %
Injection with phenylhydrazine hydrochloride	non treated	8 2	2 2	81 2	2 7	–	–	–	–
	3rd day after injection	8 9	43 3	87 5	51 7	–	–	–	–
Bleeding	non treated	9 9	1 9	85 2	1.5	87 6	2 7	35 6	2 0
	7th day after starting bleeding	11 3	48 9	93 1	47 7	91 7	39 3	47 3	53 4

Thus the catalase activity per cell decreased by about 58% by the maturation of reticulocytes to the red cells In the case of the bloods of two acatalasemic mice examined, catalase activity per cell also decreased by about 96% by the maturation of reticulocytes

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FEINSTEIN [9] reported that catalase of mature erythrocytes of acatalasemic mouse is less stable to heat than that of normal mouse erythrocytes. The present observation also shows that catalase of reticulocytes of acatalasemic mouse is also less heat stable than that of normal reticulocytes. Data suggest that catalase in acatalasemic red cells is actually the same as that of reticulocytes from the same mouse and the enzyme is different from normal catalase in the molecular structure. The suspected changes in the molecular structure of the catalase of acatalasemic and homozygous hypocatalasemic mouse mutants will be due to the mutation of the structural gene and the formation of defective enzyme.

In Japanese acatalasemia, the catalase activity is very low, but the catalase is heat stable and does not show any difference from the catalase of normal humans. The mechanism of the development of the Japanese acatalasemia is completely obscure at present but some defectiveness in the process of gene expression is suspected.

The nature of catalase in heterozygous hypocatalasemic mice has not yet been confirmed, but the present experiment revealed the heat stability in between the catalase of non-acatalasemic and acatalasemic mice and the similar degradation rate to the normal catalase. Above results elucidate that the catalase from heterozygous hypocatalasemic mice has actually the same character as that from normal mice, and it is different from the catalase of acatalasemic and homozygous hypocatalasemic mice, as far as degradation rate is concerned. As the catalase in heterozygous hypocatalasemic mice shows a less heat stability than normal catalase, it is reasonably supposed that catalase from hypocatalasemic individual is possibly consisted of normal catalase in a large amount and of acatalasemic catalase in a small amount, though further investigation will be necessary for more detailed explanation.

Acknowledgements. We thank Dr. S. SENO, Professor of Pathology, Okayama University Medical School, for discussion and criticism of this manuscript.

Summary

In order to clarify the enzymatic property of blood catalase from the acatalasemic and hypocatalasemic mice, changes in the enzymatic activity during maturation of reticulocytes and the heat stability were observed both in normal and anemic conditions. Observations revealed that the catalase activity in acatalasemic mice is very low in reticulocytes and yet

its degradation by maturation is considerably great In homozygous hypocatalasemic mice, the catalase activity in reticulocytes is relatively high comparing to that of acatalasemic one, but the degradation rate of the enzyme by maturation is nearly the same as that of acatalasemia Heat stability test of the enzyme suggested the catalase from acatalasemic reticulocytes is different from normal catalase in the molecular structure

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Blastentransformation und DNS-Synthese in Lymphozytenkulturen von Patienten mit aplastischer Anämie (Panmyelopathie)[1]

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Die aplastische Anämie (AA) ist durch eine Verminderung der hämopoetischen Zellen des Knochenmarks in allen drei Zellsystemen gekennzeichnet (Panmyelopathie). Während für die sogenannten sekundären Formen der AA als Ursache ionisierende Strahlen, Zytostatika, chemische oder infektiöse Noxen und neoplastische Prozesse in Frage kommen, ist die Ursache der «idiopathischen» Formen unbekannt. Eine Reihe von Befunden weist darauf hin, dass immunologische Vorgänge in der Pathogenese der AA eine Rolle spielen könnten. So werden im Knochenmark von Patienten mit AA und positivem Coombs-Test ausgeprägte Infiltrationen von Lymphozyten und Plasmazellen beschrieben [1]. Weiterhin sind in einer Reihe von Fällen mit AA Antikörper gegen Erythrozyten, Leukozyten und Thrombozyten [2] sowie ein positives LE-Zellphänomen und antinukleäre Antikörper [3] beschrieben worden. Während diese Befunde auf die Beteiligung humoraler Antikörper in der Pathogenese der AA hinweisen, könnte die starke lymphatische Infiltration des Knochenmarks von manchen Patienten mit AA als Hinweis für eine zelluläre Immunreaktion nach Art einer Lymphozyten-Targetzell-Interaktion gedeutet werden. Die *in vitro*-Reaktion von Lymphozyten auf unspezifische und *spezifische Stimuli wird allgemein als eine Ausdrucksform der zellulären* Immunitätslage angesehen. In den vorliegenden Untersuchungen wurden, wie bereits vorläufig mitgeteilt [4], Blutlymphozyten von

[1] Die Untersuchungen werden unterstützt im Rahmen der Euratom-Verträge Nr. 072-68-1 BIO D und 079-69-1 BIA C.

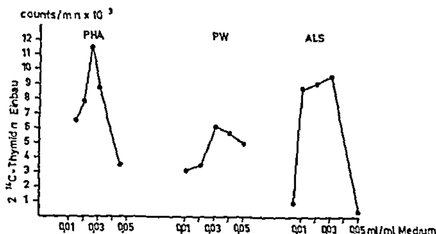


Abb 1 DNS-Synthese von Blutlymphozyten einer normalen Versuchsperson nach 3tägiger Stimulation *in vitro* mit verschiedenen Dosen von Phytohämagglutinin (PHA), Pokeweed Mitogen (PW) und Antilymphozytenserum (ALS) Die aufgetragenen Punkte stellen Mittelwerte von 4 Kulturansätzen dar

Patienten mit idiopathischer und medikamentös induzierter AA mit Phytohämagglutinin (PHA), Pokeweed Mitogen (PW) und Antilymphozytenserum (ALS) stimuliert und die Blastentransformation und DNS-Synthese dieser Zellen bestimmt

Material und Methoden

1 Phytohämagglutinin (PHA) Wir verwendeten PHA der Firma Burroughs Wellcome and Co, London (Batch No K 8581) Die optimale Stimulation lag bei einer Konzentration von 0 025 ml für 10⁶ Lymphozyten/ml Kulturmedium (Abb 1)

2 Pokeweed Mitogen (PW) Aus den Wurzeln der *Phytolacca Americana* wurde nach der Methode von Börjeson *et al* [5] ein phosphatgepufferter Kochsalzextrakt hergestellt Der Extrakt wurde durch negativen Druck 5fach konzentriert und enthielt dann 0 5 mg Protein pro ml [6] Zur Stimulation von 10⁶ Lymphozyten in 1 ml Kulturmedium erwies sich eine Menge von 0 03 ml PW als optimal (Abb 1)

3 Antilymphozytenserum (ALS) Kaninchen wurden durch dreimalige i v Injektion von je 10⁸ Blutlymphozyten eines Patienten mit chronischer lymphatischer Leukämie in Abständen von 14 Tagen immunisiert Den Tieren wurde am 10 und 13 Tag nach der letzten Injektion Blut entnommen und das Serum mehrfach bei 4°C und 37°C mit menschlichen Erythrozyten absorbiert. Der zytotoxische Titer des absorbierten Serums betrug 1 512 der Agglutinationstiter 1 1024 Eine Konzentration von 0 025 ml ALS pro 10⁶ Lymphozyten pro ml Kulturmedium ergab eine maximale Stimulation (Abb 1)

4 Lymphozytenkulturierung Es wurde eine Modifikation der Methode von Greaves *et al* [7] verwendet Den Patienten wurden 60–80 ml heparinisiertes Venenblut entnommen

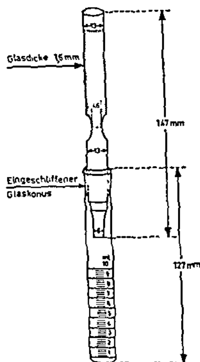


Abb. 2 Modell einer mit Nylonfasern gefüllten Glassäule zur Abtrennung der Granulozyten und Monozyten von Lymphozyten.

(5 USP Einheiten Heparin[1] pro ml Blut) und die Erythrozyten durch Inkubation bei [illegible]

resuspendiert und nochmals 7 min bei 225 g zentrifugiert. Bei Dextranzusatz wurden die Zellen ein weiteres Mal gewaschen. Die Zellen wurden danach in 2–4 ml komplettem Medium resuspendiert (komplettes Medium enthält MEM Eagle[4], 1% 200 mM L-Gluta-

[1] Liquemin, Firma Hoffmann La Roche AG, Grenzach/Baden.

[2] Dextran 250, Firma Pharmacia Uppsala, Schweden. Verwendet wurde Dextran 5% in physiologischer Kochsalzlösung

[4] Minimum Essential Medium Eagle without L-Glutamin, for Suspension Cultures, Grand Island Biological Company, Grand Island, N.Y., USA.

[5] Penicillin Streptomycin Mixture, 5000 Units each per ml, Firma Microbiological Associates Inc., Bethesda, Md., USA.

min[4], 1% Antibiotikalösung[5] und 20% inaktiviertes fötales Kälberserum[7]) Die Granulozyten und Monozyten wurden von den Lymphozyten durch Inkubation in einer mit Nylonfasern[8] gefüllten Glassäule (Abb 2) und nachfolgende Passage abgetrennt. Man erhält so eine 85–95% Lymphozytensuspension Nach Zellzählung und Differenzierung wurde die Zellsuspension auf eine Konzentration von 10^6 Lymphozyten/ml komplettes Medium eingestellt und in Mengen von 1 ml in Bijoux Flaschchen[9] abgefüllt Den Kulturen wurde PHA, PW bzw ALS entsprechend den vorher bestimmten optimalen Konzentrationen zugesetzt Bestimmung des 2-^{14}C-Thymidin Einbaues und morphologische Auswertung der jeweils dreifach angesetzten Kulturen erfolgte nach 1, 2, 3 oder 4 Tagen

5 Morphologische Auswertung Präparate der Zellkulturen wurden mit Hilfe einer Zytozentrifuge[10] hergestellt Die Zellsuspensionen wurden hierzu im Verhältnis 1 1 mit physiologischer Kochsalzlösung verdünnt und Mengen von 0,3 ml dieser Suspension 5 min bei 850 U/min zentrifugiert Die Präparate wurden luftgetrocknet und 10 min in absolutem Methanol fixiert und nach Pappenheim gefärbt 500 Zellen wurden ausgezählt und der Anteil der transformierten Zellen («transitional cells», Blasten, Mitosen) und der kleinen Lymphozyten in Prozent angegeben

6 Bestimmung des 2-^{14}C-Thymidin Einbaus 12 bis 16 Stunden vor der «Ernte» wurde den Kulturen 2-^{14}C-Thymidin[11] in einer Konzentration von 0,031 µC/ml (spezifische Aktivität 35,6 µC/mMol) zugesetzt Die Weiterverarbeitung der Kulturen für die Radioaktivitätsmessung im Flüssigkeitsscintillationszähler[12] erfolgte nach der Methode von LISO und HOLT [8]

Patientenauswahl 11 Patienten mit aplastischer Anämie wurden untersucht Die Diagnose basierte auf den folgenden Kriterien 1 verminderte Zahl der peripheren Erythrozyten, Leukozyten und Thrombozyten, 2 Hypoplasie aller 3 Zellsysteme in einem durch Stanzbiopsie gewonnenen Knochenmarkzylinder, 3 Ausschluss einer für die Pancytopenie verantwortlichen Allgemeinerkrankung

7 Patienten boten in ihrer Anamnese keinen Hinweis für eine durch Medikamente oder Chemikalien induzierte Knochenmarksschädigung 4 Patienten hatten grosse Mengen von Analgetika, Trenimon bzw Chloramphenicol erhalten 8 normale Versuchspersonen dienten als Kontrollen

Ergebnisse

Morphologische Auswertung. Abbildung 3 zeigt den Prozentsatz transformierter Zellen PHA-stimulierter Lymphozytenkulturen von 6 Patienten ohne Medikamentenanamnese und von 6 Kontrollpersonen. Am 2. Tag nach Kulturbeginn waren signifikant mehr Lymphozyten von Patienten transformiert ($61 \pm 9\%$) als von gesunden Versuchspersonen ($26 \pm 10\%$; $p < 0{,}005$). Am 3. Tag war der Unterschied nur noch gering ausgeprägt ($p < 0{,}1 > 0{,}05$). Abbildung 4 zeigt als

[5] L-Glutamin 200 mM Solution, Firma Microbiological Associates Inc., Bethesda, Md USA

[7] Fetal Calf Serum, Firma Microbiological Associates Inc, Bethesda, Md., USA

[8] Leuko-Pak, Firma Fenwal Laboratories, Morton Grove, Ill, USA

[9] Bijoux-Fläschchen, Firma Gallenkamp, London, England

[10] Zytozentrifuge, Firma Shandon, Shandon Labortechnik GmbH, Frankfurt/M

[11] 2-^{14}C-Thymidin von Radiobiochemical Centre, Amersham, England

[12] Packard Tri-Carb, Liquid Scintillation Spektrometer, Modell 3380

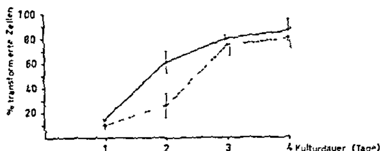


Abb. 3 Prozentsatz transformierter Zellen von 6 Patienten mit «idiopathischer» aplastischer Anämie (——) und 6 Normalpersonen (- - -) nach Stimulierung mit Phytohämagglutinin

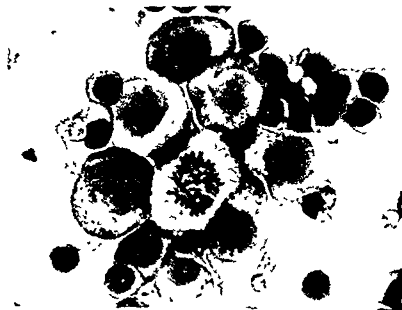


Abb. 4 Beispiel einer mit PHA stimulierten Lymphozytenkultur. Man sieht eine Mitose, mehrere Blasten, «transitional cells» und kleine Lymphozyten

Beispiel einer PHA-stimulierten Kultur mehrere transformierte Zellen «transitional cells», Blasten, Mitosen) im Vergleich zu kleinen Lymphozyten

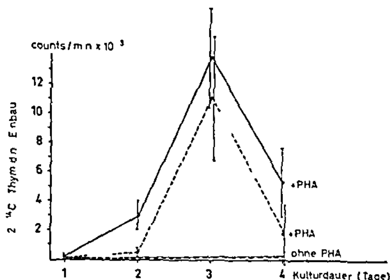


Abb 5 Stimulierung der DNS-Synthese von Lymphozyten durch Phytohämagglutinin (PHA) bei 7 Patienten mit «idiopathischer» aplastischer Anämie (——) und 8 Normalpersonen (----)

2 ¹⁴C-*Thymidin-Einbau* a) Patienten ohne Medikamentenanamnese Die Verlaufsformen von PHA-stimulierten Lymphozytenkulturen zeigten, daß auch hier am 2 Tag nach Kulturbeginn bei Patienten der 2-¹⁴C-Thymidin-Einbau höher lag als bei Kontrollpersonen (Abb 5) Der Unterschied war signifikant ($p < 0,005$) Am 3 Tag lag der Durchschnittswert bei Patienten ebenfalls hoher als bei normalen Versuchspersonen, jedoch war der Unterschied wegen der grossen Streubreite nicht signifikant ($p = 0,1$) Entsprechende Ergebnisse zeigten sich bei Verlaufskurven von PW- und ALS-stimulierten Lymphozytenkulturen (Abb 6)

b) Bei Patienten mit Medikamentenanamnese Abbildung 7 zeigt, dass bei 3 Patienten die starke Stimulation der DNS-Synthese am 2 Tag nach Kulturbeginn fehlt Die Lymphozytenkulturen verhielten sich somit ähnlich wie die normaler Versuchspersonen Im Gegensatz dazu war bei einer Patientin, die eine AA nach Chloramphenicol entwickelte, der 2 ¹⁴C-Thymidin-Einbau nach PHA-Stimulierung am 2 Tag deutlich erhoht

Versuche zur Stimulation der Lymphozyten mit autologem Knochenmark, mit Chloramphenicol und mit Serum Bei 2 Patienten wurde versucht, die Lymphozytenkulturen durch Zusatz verschiedener Mengen von

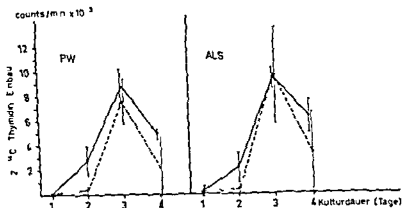


Abb. 6 2 ¹⁴C-Thymidin-Einbau von Lymphozyten von 6 Patienten mit »idiopathischer« aplastischer Anämie (——) und 8 Normalpersonen (-----) nach Stimulation mit Pokeweed Mitogen (PW) und Antilymphozytenserum (ALS)

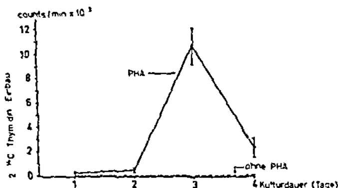


Abb. 7 Stimulierung der DNS-Synthese von Lymphozyten durch Phytohämagglutinin (PHA) bei 3 Patienten mit Medikamentenanamnese

frischem Knochenmark oder von Knochenmark-Homogenat, das durch wiederholtes Frieren und Auftauen hergestellt wurde, zu stimulieren. Eine Steigerung der DNS-Synthese der *Lymphozyten* wurde aber nicht beobachtet. Ebensowenig wurden die Lymphozyten von 2 Patienten, die Chloramphenicol erhalten hatten, durch Zusatz von Chloramphenicol in einer Konzentration von 5, 20, 100 und 300 μg/ml stimuliert

In weiteren Versuchen wurden Lymphozyten von normalen Versuchspersonen in Serum (40%) von Patienten ohne Medikamentenanamnese und Lymphozyten von Patienten mit AA in Serum (40%) von normalen Versuchspersonen mit und ohne Zusatz von PHA kultiviert. Es zeigten sich keine wesentlichen Unterschiede in der Stimulierung der Lymphozyten unter den verschiedenen Bedingungen.

Diskussion

Die vorliegenden Ergebnisse zeigen, dass die Lymphozyten bei 8 von 11 Patienten mit AA nach Stimulation mit PHA, PW und ALS am 2. Tag eine signifikant stärkere Steigerung der DNS-Synthese und Zahl transformierter Zellen aufweisen als Lymphozyten von gesunden *Kontrollpersonen. Dieser Befund bestätigt und ergänzt vorläufige Un*tersuchungen unserer Gruppe, in denen nur nach morphologischen Kriterien und ohne konstante Lymphozytenzahlen in den Kulturen eine gesteigerte Blastentransformation der Lymphozyten auf PHA-Stimulation festgestellt wurde [9].

Zunächst war die Frage zu klären, ob ein Faktor im Serum von Patienten mit AA die DNS-Synthese der Lymphozyten steigert. In unseren Untersuchungen wurden Lymphozyten von Patienten und normalen Kontrollpersonen mehrfach gewaschen und in Kulturen mit 20% inaktiviertem fötalem Kälberserum angesetzt, so dass eine Mitwirkung des autologen Serums nicht gegeben war. Zudem fand sich in mehreren Kulturansätzen kein signifikanter Unterschied im Stimulierungsgrad der Lymphozyten, wenn die Zellen normaler Kontrollpersonen im Serum von Patienten mit AA angesetzt wurden oder Lymphozyten von Patienten im Serum normaler Kontrollpersonen. *Danach musste die Steigerung der* DNS-Synthese in der 48-Stunden-Kultur auf einer an die Zelle selbst gebundenen Eigenschaft beruhen.

Zur Klärung für die rasche Steigerung der DNS-Synthese in Lymphozytenkulturen von Patienten mit AA kommen 2 mögliche Mechanismen in Betracht:

1) Im peripheren menschlichen Blut befinden sich 0,1% der mononukleären Zellen normalerweise in DNS-Synthese [10]. Cooper und Firkin [11] fanden eine gesteigerte Anzahl dieser Zellen bei 3 Patienten mit «idiopathischer» AA, nicht aber bei 4 Patienten, die eine toxische Schädigung des Knochenmarkes hatten. Es ist immer wieder disku

tiert worden, ob derartige DNS synthetisierende Zellen Stammzellencharakter haben [12, 13, 14]. Leider fehlt aber eine Methodik, um die Funktion solcher Zellen und insbesondere ihre Stammzellfähigkeit zu testen, so dass die Frage offenbleibt, ob eine gesteigerte Zahl solcher Zellen im peripheren Blut bei AA eine Reaktion auf ein hypoplastisches Knochenmark darstellt. Mit den hier angewandten Methoden konnte eine gesteigerte DNS-Synthese der Lymphozyten von AA-Patienten ohne Zusatz von Stimulantien nicht nachgewiesen werden, es wäre jedoch denkbar, dass bei AA eine normalerweise nicht im Blut vorkommende, andere Lymphozytenpopulation vorliegt, die nach Stimulation in der Kultur rascher mit einer Steigerung der DNS-Synthese reagiert.

b) Ein ganz anderer möglicher Mechanismus beruht auf der Hypothese, dass bei manchen Fällen von AA eine Autoimmunreaktion eine Rolle spielen könnte. Danach könnte dieselbe Lymphozytenpopulation, die *in vitro* mit einer raschen Steigerung der DNS-Synthese reagiert, *in vivo* eine zytotoxische Autoimmunreaktion gegen autologe hämopoetische Zellen des Knochenmarkes auslösen, ähnlich wie Lymphozyten von Ratten mit experimenteller Nephrosis Monolayer-Kulturen von Nierenzellen abtöten [15]. In Anwendung dieser Vorstellungen waren unsere Versuche, Lymphozyten von Patienten mit AA mit autologem oder homologem Knochenmark zu stimulieren, nicht erfolgreich. Weitere Experimente mit Knochenmarkkulturen müssen zeigen, ob Lymphozyten von Patienten mit AA zytotoxisch für diese Kulturen sind. Aus dieser Hypothese heraus wäre es auch verständlich, dass eine solche leicht stimulierbare Population mit einem raschen Anstieg der DNS-Synthese bei solchen Patienten nicht gefunden wird, bei denen die aplastische Anämie auf einer toxischen Schädigung des Knochenmarkes beruht. Dass aber auch dies kein genereller Befund ist, zeigt die Tatsache, dass wir bei einer Patientin, die nachweislich mehrfach Chloramphenicol erhalten und im Anschluss eine aplastische Anämie entwickelt hatte, einen raschen Anstieg der DNS-Synthese der Lymphozyten nach PHA-Stimulation gesehen hatten. Ob der hier beschriebene rasche Anstieg der DNS-Synthese ein Kriterium zur Abgrenzung von idiopathischer und sekundärer Form der aplastischen Anämie darstellt, muss durch weitere Untersuchungen an einem grösseren Patientengut geklärt werden.

Wir bedanken uns bei Frau A. von Neubeck und Frau J. Flad für ihre ausgezeichnete technische Mitarbeit.

In weiteren Versuchen wurden Lymphozyten von normalen Versuchspersonen in Serum (40%) von Patienten ohne Medikamentenanamnese und Lymphozyten von Patienten mit AA in Serum (40%) von normalen Versuchspersonen mit und ohne Zusatz von PHA kultiviert. Es zeigten sich keine wesentlichen Unterschiede in der Stimulierung der Lymphozyten unter den verschiedenen Bedingungen.

Diskussion

Die vorliegenden Ergebnisse zeigen, dass die Lymphozyten bei 8 von 11 Patienten mit AA nach Stimulation mit PHA, PW und ALS am 2. Tag eine signifikant stärkere Steigerung der DNS-Synthese und Zahl transformierter Zellen aufweisen als Lymphozyten von gesunden Kontrollpersonen. Dieser Befund bestatigt und ergänzt vorläufige Untersuchungen unserer Gruppe, in denen nur nach morphologischen Kriterien und ohne konstante Lymphozytenzahlen in den Kulturen eine gesteigerte Blastentransformation der Lymphozyten auf PHA-Stimulation festgestellt wurde [9].

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Zusammenfassung

Lymphozyten von Patienten mit «idiopathischer» aplastischer Anämie, medikamentös bedingter aplastischer Anämie und von normalen Kontrollpersonen wurden *in vitro* mit Phytohämagglutinin, Pokeweed Mitogen und Antilymphozytenserum stimuliert Es zeigte sich am 2 Tag der Kultur bei Patienten mit «idiopathischer» aplastischer Anämie ein stärkerer Prozentsatz transformierter Zellen und ein grösserer Einbau von 2 ^{14}C-Thymidin als bei Patienten mit medikamentös bedingter aplastischer Anämie und bei normalen Kontrollpersonen Es wird diskutiert, ob der rasche Anstieg der DNS Synthese Ausdruck einer zirkulierenden Stammzellpopulation oder einer immunologisch vorgeprägten Lymphozytenpopulation sein konnte

Summary

Lymphocytes from patients with 'idiopathic' aplastic anaemia, drug induced aplastic anaemia and from normal controls were stimulated *in vitro* with phytohaemagglutinin, Pokeweed mitogen and anti lymphocyte serum On the second day of culture, a higher percentage of transformed cells and a higher incorporation of 2 ^{14}C-thymidine was observed in cultures from patients with 'idiopathic' aplastic anaemia than in those of normal individuals It is discussed that the rapid onset of DNA synthesis in lymphocyte cultures from patients with idiopathic aplastic anaemia may represent a feature of either a circulating population of stem cells or of immunologically competent lymphocytes

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Cytogenetic Findings in Acquired Aplastic Anemia[1]

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Lawler [1] has recently reviewed the cytogenetic findings in various hematological conditions. Acquired aplastic anemia is not considered, probably because little information is available. In view of the existing interest in the so called pre-leukemia states [2–4] and that acquired aplastic anemia is considered as such [3, 4], we decided to review the information on this matter. In an isolated case report [5] aplastic anemia was associated with a G trisomy in the peripheral blood and in another 47 chromosomes were found but no karyotype analysis reported [6]. In a third patient [3] a chromosomal mosaicism in the bone marrow was present with the more numerous cell line lacking one autosome in the C group. Normal results have been described in 2 individuals [4] studied by direct chromosome analysis in the bone marrow and in 6 patients investigated by a peripheral blood culture stimulated with phytohemaglutinin [7].

The above mentioned discrepancies and the fact that none of these studies is a systematic investigation on acquired aplastic anemia prompted the performance of the present investigation in order to learn the cytogenetic characteristics of the usual type of aplastic anemia.

Material and Methods

Fifteen adult patients with typical acquired aplastic anemia were included in this study. The criteria for diagnosis was the presence of pancytopenia in the peripheral blood, [illegible]able bone marrow findings usually including marked hypocellularity and decreased

[1] Supported in part by grant No. TW 0031 from the USPHS.

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Table I Ceneral data of the group n es ga ed

Case No	Sex	Age years	Dura on of llness at t me of study mon hs	Et ology	Sta us of pat ents at time of s udy
1	F	14	12	th ner	act y
2	F	25	24	id opa h c	remiss on
3	F	35	24	id opa h c	act y
4	F	47	36	d opa h c	ac ty
5	F	67	?	id opa h c	act ity
6	F	73	24	idiopa hic	act ty
7	M	15	12	idiopa hic	remiss on
8	M	17	3	DDT	acti ty[1]
9	M	20	9	id opa h c	remiss on
10	M	21	144	d opa h c	act ty
11	M	24	3	d opa h c	a ti ty
12	M	30	36	id opa h c	remission
13	M	33	60	d opa h c	act y
14	M	40	11	th ner DDT	act ty[1]
15	M	42	37	chloramphen ol	act ty

These pa ents d ed some me af er our s udy Pos mortem was compa ble w th apla c anemia n all 3 cases

number of megakaryocy es and absence of any cy ological data sugges e of megalo-bla c anem a or mal gnancy n part cular leukem a Clin cally they had thrombocyto-pen purpura modera e to severe anem a and no l er spleen or lymph node enlargement Fever due to compl ca ng nfect ous processes was common

The *general data of the nd idu ls* stud ed can be seen n table I Cases 5,8 and 11 were at the n al s ages of he disease when the cytogenet c s ud es were perf med and pa en No 3 4 6 10 13 14 and 15 were s ud ed from 11 mon hs to 12 years af er diagnosis all ha ng shown la k of response to any form of trea men nclud ng androgens. An ex cep on is ase 10 who was n full remission w h anabol c s eroid during 7 years and re lapsed aft r on ra t ng a severe form of pulmonary tuberculosis 3 years ago and has done poorly s n e Ind idual 2 9 and 12 were n partial or full rem ss on at the t n e of ud Three pa ients ha e d ed No. 8 14 and 15 w h *po tmortem* find ngs compa ble w h apla ic anem a

Chromosome s ud es were done by d rect bone marrow anal sis follow ng the technique of Tjio and W ax (8) In all cases an a emp to s udy 20 metaphases su able for chromo-some coun ng wa made bu th could be accompl hed n only 8 cases In the res e t er to few cel were p esen or those seen were [illegible]
count because of poor morphology [illegible]
pl d el onl those cases w h a [illegible]
pe ed under low power and their n [illegible] in ea h pa ent regard ess of he a lequacy for coun ng At lea t 200 me aphase pla es were exam ned in every nd idual and ea h cell that appea ed pol ploid under low power was coun ed under

Table II Cytogenetic findings in 15 patients with aplastic anemia

Case No	No of diploid mitosis analyzed	Percent of polyploid cells[1]
1	5	–
2	12	0
3	12	9
4	2	–
5	31	3
6	20	0
7	40	6
8	7	–
9	20	1
10	40	2
11	4	–
12	3	–
13	20	2
14	20	11
15	20	0

[1] See text for criteria followed At least 200 mitosis were examined for this purpose

higher magnification The counts were frequently unsatisfactory because of poor morphol-
[illegible]
individuals 10 males and 7 females, with no primary or secondary hematological abnormalities was used as a comparison group Their age ranged from 19 to 40 years and had a similar socio-economic status to the patients one

Results and Discussion

The results of the cytogenetic investigations can be seen in table II. In all cases the majoritary cell line is diploid with a normal chromosome complement, in 5 cases (No 1, 4, 8, 11 and 12) the number of adequate metaphase plates were less than 10 Probably the most striking finding is the abundancy of polyploid cells in some individuals, particulary cases *3, 7 and 14* The study was repeated one year later in case 7, finding then a normal amount of polyploid cells Chromosome breaks of the single chromatid type were observed only in 3 of the total of 256 diploid cells analyzed This proportion of close to 1% is similar to what was found in our comparison group

The main finding of the present study is the absence of aneuploidy and of structural abnormalities in all cases investigated. It would seem that the usual type of acquired aplastic anemia in adults has no chromosomal abnormalities and therefore, the two cases reported with them [3, 5], are of great interest specially if they eventually develop leukemia, which would suggest that chromosomal imbalance does indeed leads to malignancy. In particular the case reported by ROWLEY *et al.* [3] is most interesting as the abnormality was encountered in the bone marrow which is the tissue affected in aplastic anemia. The patient described by ERDOGAN *et al.* [5] was only studied in the peripheral blood and it is not known whether the abnormality was also present in the myeloid tissue.

The increase of polyploid cells in several patients may be significant. With the method employed to depict them, the normal values are 1% or less in the hands of other investigators [9] and in our comparison group 16 of the 17 individuals had no polyploid cells and 1 had 1%. It should be mentioned that the samples in the comparison group were taken and processed during the same time the patient's were being studied and that the same individual did both groups. The only difference is that only 100 metaphase plates were examined in each of them, rather than 200. The presence of a large number of polyploid cells in the bone marrow may reflect an abnormality in the normal mitogenic process in aplastic anemia. However, cases 2 and 7 were in remission at the time of study, while patient 14 was in relapse and died shortly after. The significance of this finding is not clear to us. There was also no correlation between the number of polyploid cells and the etiology, duration of illness or evolution of it.

Summary

Fifteen adults with acquired aplastic anemia were investigated in regard to their chromosomal constitution in myeloid tissue. All 15 had a normal chromosome complement and the only possible abnormality was an increase in polyploid cells in several of the cases. However, the significance of this finding is obscure.

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Myelocyte Proliferation in Pernicious Anaemia

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Morphological abnormalities have been described at all stages of granulocyte maturation in pernicious anaemia [5, 6]. However, the two characteristic disturbances in myelopoiesis in this disease are the formation of giant metamyelocytes in the bone marrow and the presence of hypersegmented polymorphonuclear leucocytes in the peripheral blood [4]. In previous investigations into the arrest of erythropoietic cell proliferation in vitamin B_{12} deficiency, we and others [9] have noted that a high proportion of giant metamyelocytes are in DNA synthesis. This paper presents the results of a more detailed study of granulocytopoiesis in pernicious anaemia using the technique of combined quantitative cytochemistry and autoradiography. The purpose of this investigation was twofold. Firstly, to determine whether the disturbance in myelopoietic cell proliferation in this disease is similar to that described in the red cell precursors [10, 12] and secondly, to elucidate the origin of the circulating hypersegmented polymorphonuclear leucocyte which is so valuable in the detection of megaloblastic change in clinical haematology.

Methods

Bone marrow has been studied from 6 patients with untreated pernicious anaemia and 4 haematologically normal persons. Hypersegmented neutrophil leucocytes were present in the blood in all 6 patients with pernicious anaemia.

The distribution of the various myelopoietic cells in the different stages of interphase (G_1, S and G_2) was determined as described previously [10]. The sequence of techniques used is summarised in table I. Granulocyte precursors were classified adopting criteria used in conventional haematology. Although metamyelocytes of normal size were present in the

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Morphological abnormalities have been described at all stages of granulocyte maturation in pernicious anaemia [5, 6]. However, the two characteristic disturbances in myelopoiesis in this disease are the formation of giant metamyelocytes in the bone marrow and the presence of hypersegmented polymorphonuclear leucocytes in the peripheral blood [4]. In previous investigations into the arrest of erythropoietic cell proliferation in vitamin B_{12} deficiency, we and others [9] have noted that a high proportion of giant metamyelocytes are in DNA synthesis. This paper presents the results of a more detailed study of granulocytopoiesis in pernicious anaemia using the technique of combined quantitative cytochemistry and autoradiography. The purpose of this investigation was twofold. Firstly, to determine whether the disturbance in myelopoietic cell proliferation in this disease is similar to that described in the red cell precursors [10, 12] and secondly, to elucidate the origin of the circulating hypersegmented polymorphonuclear leucocyte which is so valuable in the detection of megaloblastic change in clinical haematology.

Methods

Bone marrow has been studied from 6 patients with untreated pernicious anaemia and 4 haematologically normal persons. Hypersegmented neutrophil leucocytes were present in the blood in all 6 patients with pernicious anaemia.

The distribution of the various [illegible]
G_1, S and G_2 [illegible]
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Table I The sequence of techniques used in the analysis of bone marrow

1	Marrow aspiration and *in vitro* labelling with ^{3}H TdR for $^1/_2$ h
2	May Grünwald Giemsa staining
3	Recording the position and type of myelopoietic cells on a photographic map
4	Destaining and restaining by the Feulgen method using a 45 min hydrolysis in 5 N HCl at 20°C
5	Estimation of relative DNA content by microdensitometry
6	Detection of DNA synthesis by autoradiography

megaloblastic marrows, varying numbers of abnormal metamyelocytes with very large horse-shoe shaped nuclei as well as several cells with nuclei of intermediate size were invariably present All abnormal metamyelocytes have been grouped together under the heading 'giant metamyelocyte' in this paper

Protein synthesis during normal and megaloblastic granulopoiesis was studied by incubating bone marrow with tritiated leucine (^{3}H Leu) prior to the preparation of smears as described previously [11]

For the study of the DNA contents of hypersegmented polymorphonuclear leucocytes blood smears were made from fresh venous blood taken at the time of the marrow aspiration The smears were air-dried rapidly, fixed in methanol for 10 min and stained by the Feulgen method The number of nuclear segments was counted at high magnification at the time DNA measurements were made using a Deeley pattern integrating microdensitometer (Barr & Stroud, Glasgow)

Results

Figure 1 shows the distribution of the DNA contents of promyelocyte and myelocyte nuclei in a normal bone marrow. Only a small fraction of these proliferating cells have hyperdiploid DNA contents, the majority being diploid (2 n)

Normal metamyelocytes had diploid DNA contents In all 4 normal marrows, very few metamyelocytes (<1%) had tetraploid (4 n) or intermediate DNA values and these cells were larger than their diploid counterparts

Figure 2 shows the distribution of the DNA contents of promyelocytes, myelocytes and giant metamyelocytes in 3 of the patients with untreated pernicious anaemia The proportion of hyperdiploid cells is significantly higher than normal in all 3 patients In the lowest histogram (case C B) the different myelopoietic cells are not represented in their normal proportions as 50 additional giant metamyelocytes and 30 extra promyelocytes were analysed

Table II shows the percentage distribution of promyelocytes and myelocytes in the different stages of interphase in normal bone marrow

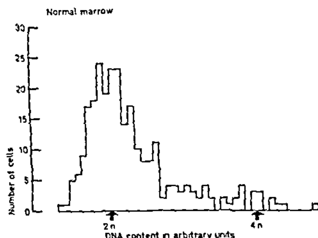


Fig 1 The distribution of the DNA contents of promyelocytes and myelocytes in normal marrow

Table II Distribution of myelopoietic cells in the various stages of interphase Pooled results of four normal marrows

Cell type	Percentages				Number of nuclei assessed
	G_1	S	G_2	U	
Promyelocyte	61	33	5	1	245
Myelocyte	62	33	4	1	552

Table III Distribution of promyelocytes in the various stages of interphase in pernicious anaemia

Case	Hb g%	WBC per mm³	Percentages				Number of nuclei assessed
			G_1	S	G_2	U	
C.B	3.6	3,500	48	46	5	1	76
T.S	3.9	4,000	54	42	4	0	71
R.P	6.4	5,800	50	41	8	2	64
I.G.	8.2	4,200	48	47	3	2	74
I.A	9.0	7,000	53	41	5	1	80

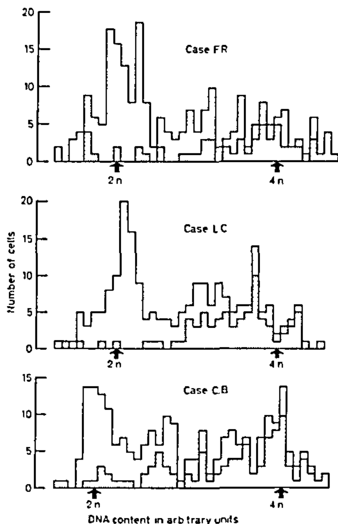


Fig 2 Histograms of the distribution of DNA contents in the proliferating population of myelopoietic cells in pernicious anaemia The stippled areas represent giant metamyelocytes

S cells were identified, after autoradiography, by their incorporation of tritiated thymidine (^{3}H-TdR) Unlabelled cells with diploid or tetraploid DNA contents were classified as cells in G_1 and G_2 respectively Column U represents cells with DNA contents between the normal spread about the 2 n and 4 n modes but which were not in DNA synthesis These may represent cells which were injured during the marrow aspiration but several other explanations are possible and have been discussed in detail previously [12]

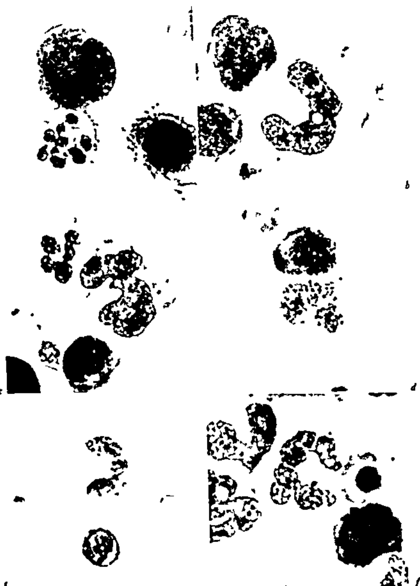


Fig. 3. Cells from bone marrow in pernicious anaemia. *a* Hypersegmented neutrophil leucocyte, 2n DNA content. *b* Giant metamyelocyte. *c* Giant metamyelocyte with bizarre nucleus. *d* Autoradiograph showing giant metamyelocyte in DNA synthesis. *e* Weakly labelled giant metamyelocyte from the same autoradiograph. *f* Segmented nucleus of a giant cell, 4n DNA content (a, b, c, f ×1,200; d, e ×800)

Table II Distribution of myelocytes and giant metamyelocytes in the various stages of interphase in pernicious anaemia

Case	Myelocytes					Giant metamyelocytes				
	Percentages				Number of nuclei assessed	Percentages				Number of nuclei assessed
	G_1	S	G_2	U		G_1	S	G_2	U	
C B	37	52	5	6	143	7	58	32	3	95
T S	40	56	4	0	205	28	55	17	0	113
R P	31	66	2	1	210	21	61	16	2	124
I C	19	78	2	1	112	17	65	16	2	86
I A	30	64	6	1	133	9	67	24	0	54

Tables III and IV show the same data for promyelocytes, myelocytes and giant metamyelocytes in untreated pernicious anaemia. A significant proportion (10–30%) of the giant metamyelocytes which were in the S compartment of interphase were very weakly labelled with ^{3}H-TdR, at a time when other granulocyte precursors, including *some giant metamyelocytes, were extremely heavily labelled* (fig 3). To avoid misinterpreting the state of these weakly labelled cells, the exposure periods of all the autoradiographs were adjusted so that several cells were always intensely labelled.

In normal bone marrow all granulocyte precursors were labelled with ^{3}H-Leu, but the grain counts over metamyelocytes were very low compared to those over the more immature cells. In megaloblastic marrows, the autoradiographs confirmed the earlier observation that several erythroblasts *did not incorporate* ^{3}H-Leu at a time when many other erythroblasts were strongly labelled and some lymphocytes showed weak labelling [11]. However, in these autoradiographs it was exceptional to find completely unlabelled myelocytes and giant metamyelocytes.

Figure 4 is a histogram of the DNA contents of circulating polymorphonuclear leucocytes in a patient with pernicious anaemia. Hypersegmented leucocytes were always diploid, both in the blood and *in the bone marrow*. However, megaloblastic bone marrows contained a few giant cells showing nuclear segmentation (fig 3) and such cells had tetraploid DNA contents.

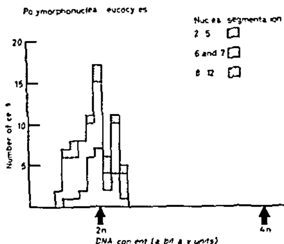


Fig 4 The distribution of the DNA contents of circulating polymorphonuclear leucocytes in pernicious anaemia (case R P)

Discussion

The ^{3}H TdR labelling index for normal human myelocytes in our *in vitro* system was 33 ± 4% This figure is consistent with previously published results which range from 13–44% [7] In the present study a similar value (33 ± 6%) was obtained for the labelling index of promyelocytes but most authors have reported higher figures [7] This discrepancy may be due to minor differences in the criteria used for cell identification or to difficulties in classifying cells when stained through an autoradiograph In this study the marrow smears were stained by a Romanowsky stain and granulocyte precursors were identified prior to autoradiography Over half the normal promyelocytes and myelocytes were in G_1 and only 4–5% in G_2 indicating a long G_1 period and a relatively short G_2 period for these cells

Former labelling studies with ^{3}H TdR *in vivo* indicated that normal human metamyelocytes do not synthesise DNA [3] The quantitative cytochemical studies reported in this paper confirm that these cells have diploid DNA contents and are a non-dividing cell class The significance of the very occasional large metamyelocyte with a hyperdiploid DNA content is uncertain as BOLL and KUHN [1] have observed repeated transformations between myelocyte and metamyelocyte

morphology in tissue culture. Results of *in vivo* studies are not consistent with such reversible changes in cell morphology, which may reflect abnormal behaviour in tissue culture.

In pernicious anaemia, the ^{3}H TdR labelling index of promyelocytes was only slightly higher than normal whilst the percentage of labelled myelocytes was about twice the normal value. If one assumes the generation time to be normal or increased the data indicate that the myelocytes have a prolonged S period in pernicious anaemia. Two other explanations are possible for this high labelling index but are considered unlikely. Firstly, a proportion of diploid myelocytes may be normally out of cell cycle (G_0 cells) and these may have been triggered into proliferation thus increasing the S compartment. A similar end result would also be produced if the duration of interphase is shortened. However, if in pernicious anaemia the myelocytes have a normal S period, the duration of interphase would have to be reduced by about 50% to account for the observed labelling indices. A study of DNA synthesis in a variety of haematological disorders does not indicate that the dynamics of myelocyte proliferation can be altered to this extent. Thus, in chronic myeloid leukaemia [2], pancytopenia associated with hypocellular, normocellular and hypercellular bone marrows, haemolytic disease of the new born, congenital erythroblastopenia [8] and primary acquired sideroblastic anaemia[1], the labelling indices are normal or show only minor variations from the normal.

Normal human metamyelocytes were not in cell cycle, whereas the giant metamyelocytes of pernicious anaemia were present in all stages of interphase. The ^{3}H TdR labelling index of giant metamyelocytes is similar to that of myelocytes. However, in 15–30% of the giant metamyelocytes the intensity of labelling was very weak indicating a gross slowing of DNA synthesis. The nucleus of the giant metamyelocyte is often abnormal being very long and ribbon like, frequently twisted and sometimes with one or more bud like projections along its length (fig. 3). In addition a high proportion of these abnormal cells are in G_2, probably indicating a pre-mitotic arrest. In view of the bizarre nuclear morphology, weak labelling with ^{3}H TdR and 'pile up' in G_2 it is very likely that the giant metamyelocyte is an effete cell which dies within the bone marrow.

[1] Unpublished observations.

The circulating hypersegmented polymorphonuclear leucocytes have a diploid DNA content and are therefore not derived from the maturation of giant metamyelocytes without cell division but must arise from the maturation of diploid metamyelocytes with normal morphology. The very occasional tetraploid bone marrow cell with a large segmented nucleus probably represents the result of continued maturation in an arrested giant metamyelocyte.

A comparison of these disturbances in myelopoietic cell proliferation with the abnormality in erythropoietic cells [12] reveals similarities. In both these cell renewal systems there is an accumulation of cells in G_2 and the defects are maximal in the most mature proliferating cell classes. The latter finding suggests that the arrest in G_2 is the result of a cumulative metabolic abnormality only manifesting itself after a certain number of cell divisions in a vitamin B_{12} deficient micro-environment. The arrest after a period in DNA synthesis, which was a prominent abnormality in the erythron, was not seen in the granulocyte precursors although there was evidence of a slowing of DNA synthesis in these cells. Furthermore, unlike the red cell renewal system where several 'proliferating' cells showed no evidence of protein synthesis, only an occasional giant metamyelocyte and myelocyte showed an arrest in protein synthesis. These differences are probably due to differences in the time an arrested cell could spend in the bone marrow prior to its loss by phagocytosis or autolysis. Granulocytes and their precursors are rich in hydrolytic enzymes and autolysis is probably a much more rapid process in these cells than in erythroblasts.



Summary

Myelocyte proliferation has been studied in normal bone marrow and in pernicious anaemia using a combination of Feulgen microspectrophotometry and autoradiography. The giant metamyelocytes show a slowing of DNA synthesis and a pile ... compartment of ... hyperseg... produced ...

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Haemolytic Disease of the Newborn due to ABO Incompatibility

A New Aid to Diagnosis

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Natal Institute of Immunology Durban

Kochwa *et al* [2] found that certain ABO isoagglutinins, after separation with the bulk of 7 S γ-globulins from other serum proteins by anion exchange chromatography, resisted inhibition by specific soluble blood group substance (hog and horse) In a group of 53 type O mothers they found 5 with such resistant isoagglutinins Four of these mothers [illegible]
required [illegible]
ABO [illegible]
readily inhibited showed no clinical evidence of haemolytic disease Mothers with a history of severe ABO haemolytic disease and donors immunised with specific soluble blood group substances, however, all had noninhibitable isoagglutinins

The technique of Kochwa *et al* [2] was suitable for antenatal specimens but the two day delay required for anion exchange chromatography prevented rapid diagnosis in the neonatal period This paper reports that, by using 2 mercaptoethanol [4] and modifying the concentration of the serum, the delay can be reduced to a few hours

Materials and Methods

Three series of specimens were used for the test

1 Antenatal samples of clotted blood from mothers (of all ABO types) with a poor obstetric history (miscarriages, abortions, stillbirths neonatal jaundice neonatal deaths and previous blood transfusions

2 Antenatal specimens selected because the results of tests on the [illegible] infants cord blood at the time of delivery were available Every effort was made to avoid selection on the basis

of the obstetric history in these cases, but medical practitioners, for obvious reasons, more often send in cord blood samples from the infants of such mothers

3 Unselected maternal blood that had been investigated for the first time at delivery only No antenatal results were available

Samples containing antibodies other than A and B isoagglutinins were excluded 2 Mercaptoethanol[1] which causes the dissociation of 19 S antibodies but has no effect on 7 S antibodies, was used to eliminate 19 S isoagglutinins from each maternal sample At a 0 1 molar concentration in saline (prepared by adding 0 56 ml to 91 4 ml of 0 89% saline) 2 mercaptoethanol showed no deterioration for up to 3 weeks at room temperature tightly stopped in a brown glass bottle

To inhibit the maternal 7 S isoagglutinins, as specific soluble blood group substances were difficult to obtain samples of saliva (collected from A and B secretors) were boiled for 20 min in a water bath After removal of the deposit by centrifuging the A and the B samples of saliva were pooled separately A convenient dilution of 1 100 in saline was selected for use confidently since tests showed that inhibition could be detected at a dilution of 1 32 000

Suspensions of enzyme-treated red cells of type A_1 and type B were used for the test After being washed twice in saline, the cells were packed and treated by adding an equal volume of 0 5% bromelin solution [3] for 10 min at 37°C followed by 3 saline washes A 2% suspension of these 'bromelin treated' cells was prepared for use

The technique for the detection of non inhibitible isoagglutinins is as follows One volume of serum was mixed with one volume of 0 1 molar 2 mercaptoethanol and incubated at 37°C for one hour Fourteen volumes of saline were then added to adjust the dilution to 1 16 One volume of the 1 16 dilution was placed in each of 2 test tubes marked 'A' and 'B' respectively To the 'A' tube one volume of 1 100 A secretor saliva was added and to the 'B' tube one volume of 1 100 B secretor saliva Both tubes were left at room temperature (±20°C) for 30 min, after which one volume of a 2% suspension of bromelin treated A_1 cells was added to the 'A' tube and of B cells to the 'B' tube After incubation at room temperature for 1 hour the results were read by lightly tapping the tubes over a Diamond light box, without prior centrifuging Tests were positive when either the 'A' or the 'B' tube or both showed obvious macroscopic cell clumping

The minimum time required to obtain a result was 2½ hours

Results

In the first series (600 maternal specimens) 87 (14 5%) had non-inhibitible isoagglutinins There were only 44 cord blood samples in this group but, as the medical practitioners had been asked particularly in each case to watch for jaundice, it is more than likely that all the affected infants were included Of these 44, 17 (2 8%) by clinical or serological standards, were suffering from haemolytic disease of the newborn, 18 were not affected though the mother/child ABO types were not homologous, and 9 had homologous ABO types.

In the second series (500 maternal specimens) 90 (18%) had non-inhibitible isoagglutinins. The results of tests on all the infants were

[1] The sample used was supplied by Fluka AG Chemische Fabrik Buchs, SG, Switzerland, and was marked 'Purum'

Table I Results obtained in an investigation for the detection of non inhibitible isoagglutinins in materna[l] sera

Series	Origin	Non inhibitible isoagglutinins			Inhibitible agglutinins		
		No	Cases with haemolytic disease of the newborn		No	Cases with haemolytic disease of the newborn	
			No	percentage		No [1]	percentag[e]
53 Group O only	Kochwa *et al* [2]	4	1	25	49	0	0
600 All ABO groups	Poor obstetric history (series I)	87	17	19 5	513	0	0
500 All ABO groups	Availability of infant cord blood (series II)	90	18	20	410	1	0 25
115 All ABO groups	Tested at time of delivery only (series III)	15	2	13 3	100	0	0

[1] Since cord blood samples were received from only a proportion of the cases, these figures must b[e] taken to indicate the number of infants whose condition justified serological investigation.

available; 18 (3 6%) suffered from haemolytic disease of the newborn; 23 were not affected though the mother/child ABO types were not homologous and 49 had homologous ABO types. One infant was affected although the maternal isolaggutinins had been inhibitible 2 months before. It is possible that at that stage the maternal isoagglutinins were 19 S and 7 S isoagglutinins developed later.

The third series (115 unselected maternal and cord blood specimens) contained 15 (13%) with non-inhibitible isoagglutinins; 2 (1.7%) of the infants suffered from haemolytic disease of the newborn

Discussion

The reasons for the various steps of the technique have been tabulated

ads

19 S isoagglutinins and does not need to be removed subsequently. 19 S antibodies do not pass the placental barrier and are therefore unlikely to be implicated in haemolytic disease of the newborn 2-Mercaptoethanol is also easy to use and is rapid in its action.

2 After the removal of the 19 S isoagglutinins, the heated samples are diluted with saline to 1:16 GROBBELAAR and GORDON [1] showed that 7 S isoagglutinins with a titre of less than 1:32 are found commonly in human serum and are of no significance to the infant Dilution at this stage ensures that at the time the red cell suspension is added, the final dilution will be 1:32

3 The samples are divided into 2 parts (one for the addition of A-secretor saliva and the other for B-secretor saliva) to investigate each isoagglutinin separately. The success or otherwise of inhibition is measured by adding a suspension of enzyme-treated cells

4 Enzyme treatment of the red cells renders them agglutinable by 7 S isoagglutinins In the test, prior treatment rather than simple addition of enzyme to the test tubes removes the possibility of additional dilution of the serum beyond 1:32 No studies by an indirect Coombs test have been done, as the results were not found to be reliable

KOCHWA *et al* [2] detected one case of haemolytic disease in 4 mothers found to have non-inhibitable isoagglutinins In our first 2 series the incidence was 1:5, and 1:7 in the third series

Since it is very probable that, regardless of cause, most infants with more than a 'normal' amount of jaundice in the neonatal period will undergo clinical and serological investigation, the figures in our 3 series can be taken to represent the maximum number of truly affected cases The one affected infant born to a mother whose isoagglutinins had previously been inhibitible supports this view

Our results show that infants affected by haemolytic disease due to ABO incompatibility are most likely to be the offspring of mothers whose isoagglutinins are non-inhibitible This supposition is in agreement with the findings of KOCHWA *et al* Moreover, modifications of technique as set out in this paper have not detracted from the value of the test and have led to an increase in the speed with which the results can be obtained

Summary

Modifications made to the original method of KOCHWA *et al* for the detection of non inhibitible A and B isoagglutinins in pregnant mothers permit results to be obtained rapidly

and easily. This makes the technique very much more useful in the diagnosis of ABO haemolytic disease of the newborn, both in the neonatal period and antenatally. Results are presented which agree with the findings of KOCHWA *et al.* that most ABO affected infants are born to mothers with non inhibitable isoagglutinins.

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Akute myelo-monozytäre Leukämie mit atypischen Naphthol-AS-D-Chloracetat-Esterase-positiven Eosinophilen

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Seit GOMORI [6] erstmals Chloracylester zum Nachweis von Esterasen verwendete, haben sich zahlreiche weitere Autoren, insbesondere in der Hamatologie, dieses Prinzips zur Esterase-Darstellung bedient. Übereinstimmend wurde festgestellt, dass ein Chloracetat abspaltendes Ferment in den Eosinophilen und ihren Vorstufen nicht nachweisbar sei [9, 10, 16, 19.] Kurzlich hat aber LÖFFLER [17] drei Fälle von akuter eosinophiler Leukose erwähnt, bei denen in den Eosinophilen Naphthol-AS-D-Chloracetat-Esterase-Aktivität nachweisbar war. LOFFLER halt diesen abnormen Befund für entscheidend bei der Diagnose einer eosinophilen Leukämie. Dass die gleiche pathologische Veränderung der Eosinophilen auch bei anderen Leukosen vorkommen kann, soll der folgende Fall zeigen.

Material und Methodik

Auf Sternal- und Blutausstriche wurden folgende färberische und zytochemische Methoden angewandt.

[illegible] Reaktion nach [illegible] ph- [illegible] Re-aktion nach LEDER [12], 6 die alkalische Phosphatase-Reaktion nach KAPLOW [8], 7. die Peroxydase-Reaktion nach LEDER [12], 8 die PAS-Reaktion und 9 die Reaktion nach ADAMS [1] in der Modifikation von LEDER *et al* [15] zur selektiven Darstellung von Eosinophilen

Fallbericht und Ergebnisse

Eine 68 Jahre alte Frau erkrankte mit schwerer Anämie und Hepatosplenomegalie bei 4600 Leukozyten, von denen nach Angaben der Klinik 88% nicht sicher differenzierbar

waren. Das uns daraufhin übersandte *Sternalpunktat* zeigte bei Pappenheim-Färbung folgenden Befund: Fettzellen stark vermindert, sehr hoher Gesamtzellgehalt. Zahlreiche [illegible]

Verminderung und schwere Kern- und Plasmaatypien der neutrophilen Granulopoesezellen. Nicht selten Pseudo-Pelgerzellen. Weiterhin zahlreiche nicht klassifizierbare Elemente. Ganz vereinzelt Auerstäbchen. Erythropoese extrem stark vermindert. Plasmazellen deutlich vermehrt. Keine Megakaryozyten.

[illegible]

werden. Die Aktivität ist an die sehr groben Granula gebunden. Im allgemeinen reagieren die reiferen Eosinophilen schwächer als die unreifen. Jedoch ist auch ein Teil der segmentierten eosinophilen Granulozyten deutlich positiv. Paramonozyten sind teils negativ, teils schwach positiv.

Beim Nachweis der alpha-Naphthylacetat-Esterase sind 21% der Zellen stark positiv und damit in Verbindung mit der typischen Zytologie als Paramonozyten ausgewiesen. Zahlreiche weitere Zellen mit gleicher Morphologie sind schwach positiv. 18% der Markzellen sind kräftig Naphthol-AS-Acetat-Esterase-positiv.

Die saure Phosphatase ist in den Eosinophilen gegenüber der Norm stark vermehrt. Die Monozyten sind mässig stark positiv, die übrigen Zellelemente zeigen etwa normale Befunde.

Alkalische Phosphatase ist lediglich in einigen Kapillarendothelien, in allen anderen Zellen einschliesslich der neutrophilen Segmentkernigen nicht zu beobachten.

Die Eosinophilen und ihre Vorstufen enthalten stark PAS-positive spezifische Granula. In den Paramonozyten eine schwach positive, teils diffuse, teils feingranuläre Reaktion.

Durch die modifizierte Adamsche Reaktion stellen sich die Eosinophilen wie im Normalfalle elektiv dar. Dabei kommt wie bei der Pappenheim-Färbung die vielfach sehr grobe Granulation deutlich zum Ausdruck. Das Plasma aller Eosinophilen ist meist vollständig mit Granula gefüllt. Zellen mit wenigen eosinophilen Granula finden sich nur sehr selten.

Fünf Tage nach der Sternalpunktion erhielten wir zur Vervollständigung der Diagnostik Blutausstriche. In diesen finden sich beim Nachweis der alpha-Naphthylacetat-Esterase 14,5% stark positive Paramonozyten, weitere 16,0% der Zellen weisen zytologisch den Habitus von Paramonozyten auf, sind aber entweder nur schwach Esterase-positiv oder negativ. Ansonsten finden sich 1% Eosinophile, 5,5% zum Teil stark atypische Neutrophile, 56,0% Lymphozyten und 7,0% nicht klassifizierbare Zellen. 13,0% aller Zellen sind stark Naphthol-AS-D-Chloracetat-Esterase-positiv. Teils handelt es sich dabei um reife Neutrophile, teils um völlig atypische Elemente.

Weitere 2 Monate später wurden uns erneut Blutausstriche übersandt. Gesamtleukozytenzahl nunmehr 50000/µl. Differential-Blutbild: nicht klassifizierbare Zellen 54,5%, Paramonozyten 30,5%, Neutrophile 2,0%, Eosinophile 1,5%, Basophile 0,5%, Lympho- [illegible]

Der Nachweis der Naphthol-AS-Acetat-Esterase ergibt den gleichen Befund wie die alpha-Naphthyl-Acetat-Esterase-Reaktion. Die wenigen Eosinophilen sind wiederum wesentlich stärker saure-Phosphatase-positiv als normalerweise.

Diskussion

Bisher wurde die Meinung vertreten, dass die Naphthol-AS-D-Chloracetat-Esterase nur in den Gewebsmastzellen, den neutrophilen Granulozyten und deren Vorstufen sowie in schwacher Aktivität in einem Teil der Blutmonozyten vorkommt. In allen übrigen Zellen des Organismus wurde das Ferment übereinstimmend nicht gefunden. Dabei wurde einhellig betont, dass insbesondere die eosinophile Granulopoiese das Enzym niemals nachweisen lasse, weder im Normalfalle noch bei Leukosen. Allein LÖFFLER [17] fand unter 225 zytochemisch untersuchten akuten Leukämien 3 eosinophile Leukosen, bei denen die Eosinophilen die gleichen Befunde wie in unserem Falle zeigten: Eine positive Naphthol-AS-D-Chloracetat-Esterase-Reaktion, die an die spezifische Granulation gebunden war (Abb. 1), eine gesteigerte saure Phosphatase-Aktivität (Abb. 2), eine positive PAS-Reaktion der spezifischen Granula (Abb. 3) und schliesslich bei der Pappenheim-Färbung grobe, tiefbasophile Granula in den frühen Vorstufen. LÖFF-

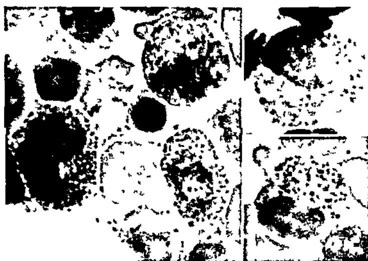


Abb. 1. Atypische eosinophile Vorstufen im Knochenmark mit positiver Naphthol-AS-D-Chloracetat-Esterase-Reaktion der spezifischen Granula (× 1400)

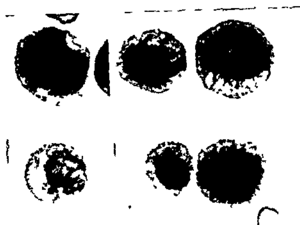


Abb. 2. Atypische Eosinophile des Knochenmarkes mit erhöhter saurer Phosphatase-Reaktion (× 1400)



Abb. 3. Atypische Eosinophile mit groben, stark PAS-positiven Granula im Knochenmark (× 1400)

LER stellt insbesondere die positive Naphthol AS-D Chloracetat-Esterase-Reaktion der Eosinophilen als typisches und entscheidendes Resultat heraus, mit dessen Hilfe man bisher am eindeutigsten eine Eosinophilenleukämie diagnostizieren und von nicht neoplastischen eosinophilen Reaktionen unterscheiden könne.

Unser Fall bestätigt die Ergebnisse von LÖFFLER. Er zeigt darüber hinaus, dass völlig identische Veränderungen der Eosinophilen auch bei anderen Myelosen vorkommen. Nach den zytologischen, zytoche-

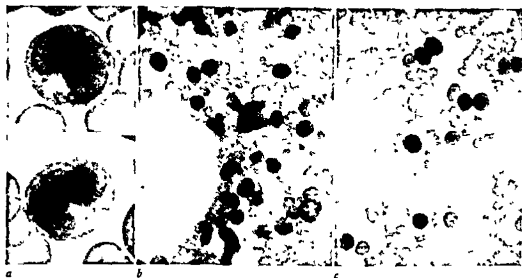


Abb. 4. a Paramonozyten aus dem peripheren Blut. Alpha-Naphthyl-Acetat-Esterase-Reaktion. Beachte die typischen, ausgeprägten Faltungen und Knickungen der Kernmembranen. *b* Paramonozyten im Knochenmark, und *c* im Blutausstrich mit positiver Naphthol-AS-Acetat-Esterase-Reaktion (*a* × 1400, *b* und *c* × 350)

mischen und quantitativen Ergebnissen liegt bei unserer Beobachtung eine myelo-monozytäre Leukämie mit einer auf das Knochenmark beschränkten erheblichen Eosinophilen-Beteiligung vor.

Wir begründeten unsere Diagnose einer myelo-monozytären Leukämie nach früher von uns [12] aufgestellten Kriterien: Die Sternalmark- und Blutausstriche, letztere insbesondere bei der zweiten Untersuchung, in der leukämischen Phase des Krankheitsbildes, weisen reichlich Paramonozyten mit den entsprechenden typischen Kernformanomalien und positiver unspezifischer Esterase-Reaktion auf (Abb. 4). Es besteht also nicht der geringste Zweifel, dass die Monozyten am leukämischen Krankheitsbild beteiligt sind. Andererseits besteht kein Zweifel an der Beteiligung der neutrophilen Reihe. Dies zeigt die positive, im Unterschied zu den Eosinophilen feingranuläre Reaktion zahlreicher, schwer atypischer Zellen beim Nachweis der Naphthol-AS-D-Chloracetat-Esterase (Abb. 5), das Vorkommen der Pseudo-Pelgerzellen und der ausserordentlich hohe Prozentsatz (87,5%) Peroxydase-positiver Zellen (Abb. 6). Dieser letzte Befund zeigt, dass die 54,5% nicht klassifizierbaren Blasten des Pappenheim-Präparates sämtlich als myeloische Zellen angesehen werden müssen. Auch geht aus diesem Befund hervor, dass Peroxydase und Naphthol-

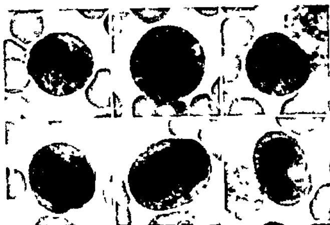


Abb. 5 Blasten sowie eine Pseudo-Pelgerzelle beim Nachweis der Naphthol AS-D-Chloracetat-Esterase im Blutausstrich (× 1400)



Abb. 6 Ein Eosinophiler (links oben) sowie mehrere Blasten im Blutausstrich beim Peroxidase-Nachweis. Zwei der Blasten (links unten und Mitte oben) enthalten je ein positives Auerstäbchen (× 1400)

AS-D Chloracetat-Esterase keineswegs übereinstimmend verteilt sind, [illegible]

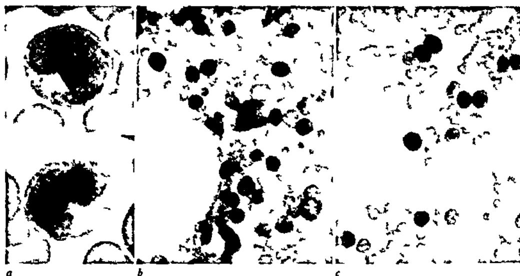


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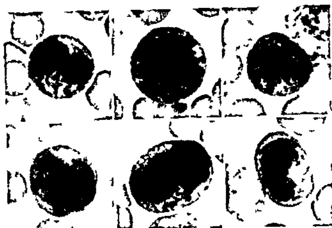


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Abb. 6 Ein Eosinophiler (links oben) sowie mehrere Blasten im Blutausstrich beim Peroxydase-Nachweis. Zwei der Blasten (links unten und Mitte oben) enthalten je ein positives Auerstäbchen (× 1400)

AS-D-Chloracetat-Esterase keineswegs übereinstimmend verteilt sind, denn bei dem letzten Fermentnachweis wurden viel weniger positive Zellen gefunden. Schliesslich sprechen auch die vereinzelt nachgewiesenen Auerstäbchen für eine granulozytäre Beteiligung an der Leuk-



Abb. 7. Atypische Eosinophile des Knochenmarkes bei der Adamsschen Färbung. Die Granula sind zum Teil recht grob, reagieren aber regelrecht (× 1400).

ämie, da sie bekanntlich pathologische Promyelozytengranula darstellen [4, 5]. Dass im vorliegenden Fall nicht von einer eosinophilen Leukämie gesprochen werden kann, geht bereits aus den beiden Differentialblutbildern hervor, in denen nur 1,0% bzw. 1,5% Eosinophile *gefunden wurden.*

Man konnte die Frage aufwerfen, ob die von uns als Naphthol-AS-D-Chloracetat-Esterase-positive Eosinophile angesehenen Knochenmarkszellen diese Bezeichnung auch zu Recht verdienen oder ob nicht atypische neutrophile Granulopoiese-Zellen mit missgebildeten eosinophil-färbbaren Granula, etwa im Sinne der Pseudoeosinophilen des Kaninchens, vorliegen. Diesem möglichen Einwand ist aber entgegenzusetzen, dass bereits im Pappenheim-Präparat kein Zweifel an der eosinophilen Natur der Zellen besteht. Vor allem aber fiel die *für Eosinophile spezifische, auf dem Tryptophangehalt der Granula* beruhende Adamssche Reaktion eindeutig positiv aus (Abb. 7).

Das wesentliche und, wie wir sehen werden, zunächst nicht losbare Problem des Falles ist das Zustandekommen des Naphthol-AS-D-Chloracetat-Esterase-Gehaltes der eosinophilen Granula. LÖFFLER diskutiert die Moglichkeit einer Änderung der Membranfunktion der Granula im Sinne einer erhohten Durchlassigkeit für das angebotene Substrat Naphthol-AS-D-Chloracetat. Diese Hypothese setzt voraus, *dass die eosinophilen Granula auch im Normalfalle das Ferment enthalten* und dass nur eine besondere Funktion ihrer Membran, die sich von den Membranen anderer Granulaarten elektronenoptisch

nicht unterscheidet [2 7 18] seinen zytochemischen Nachweis nicht erlaubt

Die von LOFFLER diskutierte Hypothese wirft damit die Frage auf ob bei den in der hamatologischen Zytochemie verwendeten lufttge trockneten und anschliessend im Falle der Naphthol AS D Chloracetat Esterase alkoholfixierten Präparaten die physiologische Membran funktion uberhaupt eine Rolle spielt Wir glauben dies nicht sondern halten es sogar fur moglich dass der zytochemische Nachweis vieler Fermente gerade darauf beruht, dass die Membranen zerstort und für Substrate durchlassig werden Unter diesem Gesichtspunkt scheint uns die Hypothese einer veranderten Membranfunktion keine wesentliche Stutze zu finden wenn sie auch keineswegs schlussig widerlegt ist

Manches deutet darauf hin dass bei den Chloracetat Esterase positiven Eosinophilen eine gegenuber der Norm veranderte stoffliche Zusammensetzung der Granula vorliegt, so die gesteigerte saure Phosphatase Aktivitat und vor allem die positive PAS Reaktion Moglicherweise beruht d es auf einer mit dem leukamischen Prozess im Zusammenhang stehenden Veranderung der genetischen Informationsmechanismen im Sinne des «one gene one-enzyme» Konzeptes von BEADLE und TATUM [3] Nach diesem Konzept wird die Produktion eines jeden Enzyms von einem bestimmten Gen kontrolliert Nach den heutigen Kenntnissen der allgemeinen Genetik muss angenommen werden dass alle Zellen des Organismus uber die potentielle genetische Information zur Bildung der Naphthol AS D Chloracetat Esterase verfügen dass aber beim Gesunden mit Ausnahme der normalerweise positiven Zellen diese Information durch einen Repressor Effekt nicht zum Tragen kommt Angesichts der zahlreichen bei Leukosen nachgewiesenen chromosomalen Aberrationen ist es nicht von der Hand zu weisen dass bei unserem und auch bei den LOFFLERschen Fallen eine Storung des Kontrollmechanismus des Chloracetat Esterase Gens bei den Eosinophilen vorliegt durch welche der abnorme Befund zu stande kommt

Dieser abnorme Befund zeigt im ubrigen gewisse Ähnlichkeiten mit den Verhältnissen in der neutrophilen Granulopoiese So nimmt im grossen und ganzen die Naphthol AS D Chloracetat Esterase Aktivitat der eosinophilen Granula mit der Ausreifung ab oder schwindet gänzlich Entsprechende Verhältnisse finden wir in der normalen neutrophilen Granulopoiese auch Zwar sind hier alle Entwicklungssta

dien fermentpositiv, jedoch die reifen Neutrophilen wesentlich schwächer als die Promyelozyten.

Angesichts der Naphthol-AS-D-Chloracetat-Esterase-positiven Eosinophilen drängt sich der Gedanke auf, ob der Befund auf eine normalerweise vorhandene gemeinsame Stammzelle für Neutrophile und Eosinophile hindeutet. Wir möchten hier grosste Zurückhaltung üben, da wir es grundsätzlich ablehnen, aus pathologischen Befunden auf normale Entwicklungsvorgänge zu schliessen [13]. Im normalen Knochenmark haben wir bisher keine Zusammenhänge zwischen eosinophiler und neutrophiler Granulopoiese gefunden [14]. Im vorliegenden Fall liessen sich bei der Adamsschen Färbung vereinzelt Zellen auffinden, die nur wenige eosinophile Granula enthielten, die also als Übergänge zwischen nicht spezifischen Promyelozyten und eosinophilen Myelozyten angesehen werden könnten. Aber es bleibt die Frage offen, ob solche Zellen im Verlaufe ihrer weiteren Entwicklung tatsächlich eine vollständige Granulation entwickeln würden oder nicht. Im zweiten Falle wäre die Granulaarmut nämlich Ausdruck eines fortschreitenden Verlustes der Granula im Sinne einer sog. Entdifferenzierung, und man konnte sich vorstellen, dass schliesslich uncharakteristische Blasten im Sinne von ROHR [20] entstehen. Für die zweite Annahme spricht der Befund, dass die gering granulierten Eosinophilen oft sehr klein waren und überdies ein schmales Protoplasma aufwiesen. Damit erinnerten sie, abgesehen von der spärlichen Granulation, eher an sogenannte Mikromyeloblasten als an typische Übergänge zwischen Promyelozyten und eosinophilen Myelozyten. Immerhin muss zugegeben werden, dass trotz unseren Bedenken der abnorme Enzymbefund Ausdruck einer gemeinsamen Stammzelle von Neutrophilen und Eosinophilen sein konnte. Vollig gleichwertig ist diesem Aspekt aber die Hypothese an die Seite zu stellen, dass lediglich eine dem Leukoseprozess zugeordnete Atypie vorliegt.

Der positive Chloracetat-Esterase-Befund in den Eosinophilen der Fälle von LOFFLER und uns zeigt schliesslich ein weiteres Mal, dass ohne eine gediegene morphologische Basis die Zytochemie in der Hämatologie nicht fruchtbringend angewandt werden kann. Wie LÖFFLER [17] sind auch wir – ganz besonders im Hinblick auf den vorliegenden Fall, bei dem z. B. eine Naphthol-AS-D-Chloracetat-Esterase-Positivität in Granulopoiese-Zellen nicht bedeutet, dass die Zellen auch zur neutrophilen Reihe gehoren – einmal mehr zu der Überzeugung gelangt, dass die Zytochemie eine wertvolle Ergänzung und ein nicht mehr

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Zusammenfassung

Es wird ein mit zahlreichen zytochemischen Methoden durchuntersuchter Fall von myelomonozytärer Leukose beschrieben, bei dem sich entgegen der Norm Naphthol AS-D-Chloracetat Esterase-positive Eosinophile finden liessen. Diese abnormen Eosinophilen zeigten auch eine positive PAS-Reaktion der Granula und eine verstärkte saure Phosphatase Reaktion. Die Bedeutung dieser Befunde wird diskutiert, wobei auf eine mögliche Änderung der Membran-Funktion der eosinophilen Granula [illegible]

Summary

A case of acute myelomonocytic leukemia subjected to many different cytochemical methods is described. In contrast to their normal behaviour the eosinophilic granulocytes and their precursors showed a positive naphthol AS-D chloroacetate esterase reaction. Moreover, there was an increased acid phosphatase reaction and a strongly positive PAS reaction of the specific granules. These observations are discussed with respect to a possible change of membrane function of the eosinophilic granules and to the possibility of a genetic defect related to the leukemic process. In addition, some comments are given as to the question whether or not a normal relationship between eosinophilic and neutrophilic cell strains can be deduced from these findings. Finally, there was no correlation or parallelity between peroxidase and naphthol AS-D chloroacetate esterase activities of the immature blasts of the case.

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Summary

A case of acute myelomonocytic leukemia subjected to many different cytochemical methods is described. In contrast to their normal behaviour the eosinophilic granulocytes and their precursors showed a positive naphthol AS-D chloroacetate esterase reaction. Moreover there was an increased acid phosphatase reaction and a strongly positive PAS reaction of the specific granules. These observations are discussed with respect to a possible change of membrane function of the eosinophilic granules and to the possibility of a genetic defect related to the leukemic process. In addition some comments are given as to the question whether or not a normal relationship between eosinophilic and neutrophilic cell strains can be deduced from these findings. Finally there was no correlation or parallelity between peroxidase and naphthol AS-D chloroacetate esterase activities of the immature blasts of the case.

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E. GRACI L (Hrsg) Internationales Symposium über die Anwendung der Vin Alkaloide Velbe und Vincristin. Urban & Schwarzenberg München 1969 223 30f fg DM 42

Th s liberally illustrated sympos on volume conta ns 39 papers and short commun tions by authors of 23 ma nly cont nental European hosp tals and cancer research cen Th rty papers are pr nted in German 5 n French and 4 only in English A large numbe presentat ons deals w th the cl n cal exper ence of the two v nca alcalo ds (Vinblast ne Vincrist ne) in the treatment of acute leuken ias mal gnant lymphomas and certa n tumors such as mammary cancer chorioep thel oma as well as tumors of the female product e sys em Several extens e discuss on parts clarify and enrich the formal prese t ons Due to the number of commu cat ons repet tions are numerous D ers ty of th peut c schedules and response cri eria unfortunately l m t con parat e conclus ons In est ng data about the pos ble mode of act on of the vinca drugs are presented by Lac

Due to the men ioned therapeut c heterogene ty the volume is of interest and value to internist exper enced in chemotherapy rather than to the general med cal public

H J SENN

The Hemophil ac and His World L univers de l'Hémophile Proc 5th Congr World Hemoph l a Mon real 1968 Editor R GOUREVEAU B bl haemat No 34 Ka Basel Munchen New York 1969 VIII+214 p 38 f g 46 tab sFr 56 US$ 1 DM 5C 332 s

Various aspects of the haem ph l a problem have been treated during the 5 h Con of the World Federa ion of Hem ph l a held in August 1968 in Montreal The first p the Congress has been devoted to the character zat on and cl n cal use of factor VIII factor IX concentra es developed for the spec fic therapy of haemoph lia A and B resul s obta ed in var ous centers d ffer w h respect to half l fe time *in vivo recovery* d ra ion of cl cal effect. However the h gh degree of efficiency has been recogn ze all in estigators. The value of the prophylact c treatment in haemophilia A has iscussed Here also the opin ons are qu te d ergent some are in fa our of a re prophylactic treatment other prefer to reserve the infus on for m anifest or m ost pro *haemorrhages*. The other chapters gi e information on the non-specif c therapy of ha of l a and of

e not conce ed as a pract cal guide for the of haemoph lic patients. However they are valuable for the physicians in charge of pa ien s

F DUCKERT

D A ... L'Hémophilie Les cahiers Bal ère Bal ère Paris

Dans ce cahier l a eur donne un aperçu rapide de l'hémophilie pour s arrêter long emps sur les aspects cliniques et thérapeutiques de cette maladie Les d ers l'hémorragie sont passés en revue illustrés d exemples frappants Un chapitre est consacré à l examen d un sujet suspect d'hémophilie La partie réservée au tra te

indique les diverses possibilités, traitement local, substitution emploi des fractions coagulantes concentrées L'auteur indique les incidents ou accidents possibles au cours du traitement substitutif Chaque type d hémorragie est considéré séparement, une place importante est faite aux hemarthroses et à la reeducation des articulations et de la musculature Les traitements associés antibiotiques antalgiques sont discutés L emploi d antifibrinolytiques est recommandé dans les cas d hématurie (Ces medicaments sont pour le moins discutables et peuvent entrainer des inconvénients graves?) Citons encore quelques règles à observer dans les cas d hemophilie compliques par la presence d inhibiteurs, des remarques sur les vaccinations et les problèmes medico-sociaux

Ce cahier est fort instructif d un point de vue pratique et clinique et donne des informations d une valeur certaine F Duckert, *Bâle*

Pathology of Erythroblastic Mitosis in Occupational Benzenic Erythropathy and Erythremia Editors F G Rondanelli P Gorini, G Gerna, and F Magliulo Bibliotheca Haematologica No 35 Karger, Basel/München/New York 1970 X+188 p 44 fig sFr 63 –/US $ 15 10/DM 63 –/126 s

Die Autoren beschreiben morphologische Untersuchungen über Mitose- und Chromosomenanomalien von Erythroblasten nach Exposition mit Benzol Die Anomalien der Teilungsfiguren von 4 Patienten mit morphologischen Anomalien der Erythroblasten nach beruflicher Benzolexposition wurden zu einem Tiermodell in Beziehung gesetzt Der Wassermolch (*Molge vulgaris* L.) zeigt nach 6monatigem Fasten bei niedriger Temperatur eine Abnahme der Blutbildung die jedoch durch Fütterung und Erwärmung (26° C) innert weniger Tage maximal aktiviert werden kann Die Autoren untersuchten die Mitosefiguren von Milzzellen dieser Tiere unter Zellkulturbedingungen und hielten die verschiedenen Teilungsstadien von Zellen mit oder ohne Benzolzusatz kinematographisch fest Die Arbeit bestätigt einmal mehr, daß Benzol zahlreiche, morphologisch erkennbare Anomalien von Chromosomen und im gesamten Ablauf der Zellteilung verursacht In der Interpretation ihrer Resultate sind die Autoren zu Recht vorsichtig Benzolexposition verursacht verschiedene Formen von Knochenmarksschädigung und scheint zu Leukämien zu prädisponieren Es bleibt aber zu beweisen, ob morphologisch erkennbare Anomalien der Zellteilung besonders der Chromosomen direkt an der malignen Entartung von Zellen beteiligt seien E. A Beck, *Bern*

Disseminated Intravascular Coagulation. Editors E F Mammen G F Anderson and M I Barnhart Schattauer Stuttgart/New York 1969

Neuere Aspekte der intravaskulären Gerinnung und Verbrauchskoagulopathie fassen die Vorträge von namhaften Spezialisten anlässlich eines Symposiums in Detroit vom Januar 1969 zusammen Die Pathogenese der intravaskulären Gerinnung Beziehungen zum Sanarelli Shwartzman Phänomen Störungen der Mikrozirkulation Gerinnungsanomalien beim Schock sowie Berichte über spezielle Formen der Verbrauchskoagulopathien interessieren in erster Linie den Kliniker Neuere Kenntnisse über Diagnostik, wie die Erfassung von Fibrinogen Abbauprodukten und Intermediärprodukten der Fibrinbildung werden ebenfalls besprochen Neueren Datums sind auch Befunde über glomeruläre Ablagerungen von Fibrin im Rahmen einer hyperakuten Abstossungsreaktion nach species-inkompatibler Nierentransplantation Die Arbeit von Riddle und Barnhart weist sehr schön die spezifische Mobilisierung der eosinophilen Granulozyten durch lokale oder generalisierte Fibrinablagerungen nach Insgesamt ist die Gestaltung des Bandes sorgfältig Ein recht vollständiges Sachverzeichnis erleichtert die Konsultation über spezielle Fragen Das Buch ist dem an Blutgerinnungsproblemen interessierten Kliniker und Experimentalpathologen wärmstens zu empfehlen E. A Beck, *Bern*

Acta haemat. 44: 65–77 (1970)

Experimental and Clinical Studies on Muramidase (Lysozyme)

I. Muramidase Activity of Normal Human Blood Cells and Inflammatory Exudates[1]

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Muramidase, although discovered over 40 years ago by Fleming and named 'Lysozyme' for its bacteriolytic activity [1], has gained considerable attention in recent years because of its possible differential diagnostic value in acute leukemias [2–5] and certain renal disorders [6–8]. The enzyme, a heat labile protein with a molecular weight of 14 000–15 000, has been isolated in chromatographically pure form from leukocytes [9] and human leukemic urine [3][2]. Muramidase is present in normal human plasma and serum, saliva, tears, monocytes, granulocytes and their precursors, as well as renal tubular cells, but seems to be absent or detectable only in trace amounts in normal spinal fluid, urine, erythrocytes, platelets and small lymphocytes [10–12]. Except for our own preliminary data on skin chamber exudates in normal man [13] and the investigations of muramidase and β-lysin in rabbit peritoneal exudates [14], we are not aware of any study of muramidase activity in experimental sterile inflammation.

This paper reports our experience with serial muramidase determinations in the course of quantitative and kinetic studies on localized leukocyte mobilization (LLM) in 52 normal subjects [13]. Muramidase was determined routinely in plasma, serum and in subsequent skin chamber exudates up to 24 hours, as well as in peripheral blood

[1] Study supported by U.S. public health service grant No. CA-5834 from the National Cancer Institute, Bethesda, Md. (USA) and research grant No. 3100'69 from the Swiss National Funds for Scientific Research.

[2] Arakawa, W. and Holland, J. F.: Unpublished data, 1967.

cells and exudate leukocyte homogenates at different times of the inflammatory process. These data were related to the kinetics and cytology of the observed leukocyte exudation in order to gain information about the markedly increased muramidase activity observed in inflammatory exudates [13, 14] and its possible significance.

Materials and Methods

Study group A total of 457 determinations of muramidase were performed in 88 untreated cell free sera or plasma samples, 284 inflammatory exudates and 85 specimens of purified populations of blood and exudate cells of 52 healthy subjects A large number of additional enzyme determinations was carried out in preliminary studies on the influence of various factors on muramidase standard curves and preservation of test samples

Technique of localized leukocyte mobilization (LLM) The plastic skin chamber technique, a simplified and more reproducible modification of a previous glass chamber approach by others [15] has been discribed elsewhere in detail [13, 16] To summarize briefly, standardized rectangular skin abrasions were performed quickly with a high speed drill The resulting sterile leukocyte (WBC) reaction was collected into small plastic skin chambers, filled initially with 2 0 ml of autologous cell free serum Periodic removal of the chamber content was performed at 2, 4, 6, 8, (12) and 24 hours after skin abrasion A new aliquot of fresh serum was then used to refill the chamber No antigens or extraneous additions were introduced into the chamber systems in this study Sterility of the tests was routinely monitored by culturing the final chamber exudate Cell recovery was expressed in millions of exudate WBC mobilized per cm^2 abrasion surface, either as cumulative total LLM in 24 hours ($WBC \times 10^6/cm^2/24$ h) or as mobilization rates ($WBC \times 10^6/cm^2/h$)

Serum and cell isolation procedures All centrifugations were done at controlled speed at 4°C in sterile, siliconized glass-ware Autologous plasma was obtained by centrifugation of heparinized or oxalated venous blood at 600 *g* for 10 min Autologous serum for the leukocyte chamber studies was obtained from 30 ml of venous blood by the same centrifugation process after allowing the sample to clot for 45–60 min at room temperature Mixed leukocytes were harvested from the WBC rich plasma supernatant of oxalated blood samples after gravity sedimentation of erythrocytes (RBC) Red blood cells were obtained from the RBC sediment of the same blood samples and were once passed through a small glass-bead [illegible]

[illegible]

mature PMN, were obtained from the leukocyte chamber exudates 2–24 hours after skin abrasion without further purification An attempt was made to obtain monocyte rich WBC populations by the RABINOWITZ' elution method [17] from the venous blood of 3 otherwise healthy volunteers with reactive (viral?) monocytosis Platelets were obtained from the oxalated plasma of the leukocyte donors after gentle removal of the WBC at 150 200 g by consecutive high speed sedimentation at 1,000 *g* for 30 min

All cell mixtures and purified cell populations assayed for muramidase were washed [illegible] The test [illegible]

sequential rapid freezing thawing cycles in dryice/methanol and in a 40°C waterbath res-

pectively, or else stored overnight at 4°C and lysed the same way 8–12 hours later. Temperature lysis was given preference to sonication, because the latter procedure has repeatedly shown incomplete cell destruction as well as enzyme recovery in preliminary comparative tests.

Muramidase assay. All specimens were assayed for muramidase after storage periods ranging from 1–32 days at −20°C. Prior to enzyme determination, specimens were centrifuged briefly to remove cellular debris. Only the clear supernatant of the cell homogenates was used for enzyme determinations. Muramidase was assayed according to a slight modification of the lysoplate method of OSSERMAN and LAWLOR [3]. Transparent square plastic dishes (Integrid 100 × 100 × 15 mm, Falcon Plastic, Los Angeles, Calif., USA) containing 36 fields were used for all determinations. The cylindric wells, cut into the soft agar-gel suspension of *Micrococcus lysodeikticus* (Worthington Biochemicals, Freehold, N.J., USA) by suction, had a volume of approximately 25 mm³ and were carefully filled with 20 mm³ micropipettes. Standard dilutions used for muramidase determinations were 1, 5, 10, 100 and 500 µg of [illegible]

or triplicate, a precaution abandoned later in the study because of excellent reproducibility of results. Reading of the enzymatic clearing zones in the hazy agar plates was performed on a magnifying viewer projector screen in arbitrary units (The National Instrument Co., Philadelphia, Pa., USA). Muramidase activity was expressed in µg of eggwhite lysozyme activity per ml for the case of serum, plasma or BSS, and in µg of enzyme activity per 10⁶ of cellular elements for RBC, WBC, PMN, monocytes, lymphocytes and per approximately 10⁹ for platelets.

Results

Influence of media on standard curves in lysoplate assay. Irrespective of the medium employed for standard dilutions of egg-white lysozyme, the standard curves usually formed a fairly straight line [illegible] of enzyme concentratio[illegible] the slope of the standa[illegible] zones plotted on the a[illegible] were dependent on the diluting medium: urine resulting in larger bacterial clearing zones than RPMI 1640 or BSS at the same enzyme concentrations. Human serum and plasma containing egg-white lysozyme gave standard curves very similar to human urine at concentrations over 50 µg/ml, where the inherent muramidase activity of these media became negligible. Urine was [illegible] [illegible] other authors [23].

Muramidase activity of normal blood cells. Figure 2 shows the median and range of muramidase activity of the divers cellular elements of normal human blood. The respective enzyme values per 10⁶ cells are:

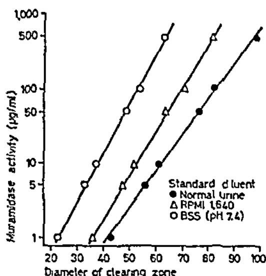


Fig 1 Influence of diverse media on muramidase standard curves (diameter of clearing zones read in artificial units)

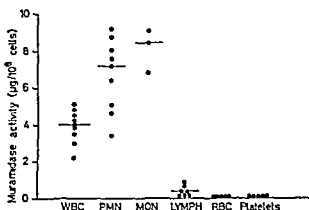


Fig 2 Muramidase activity of normal human blood cells expressed in egg white lysozyme standard equivalents *WBC* Native mixed leucocyte suspension *PMN* granulocytes *MON* monocytes *LYMPH* lymphocytes *RBC* erythrocytes (degree of purity see text)

mixed WBC = 4 0 (2 1–5 1) µg, PMN = 7 2 (3 6–9 2) µg, partially purified monocytes = 8 4 (6 5–9 1) µg, lymphocytes = 0 4 (0 1–0 9) µg, RBC and platelets = <0 1 µg (not measurable) Assayed in sufficiently pure populations – with the exception of monocytes – it seems quite clear, that PMN are the major source of muramidase in the circulating

blood The comparative role of monocytes is difficult to determine due to the problems of isolating them in sufficiently pure form from normal blood It seems, however, that monocytes on an isocellular basis, do not contain significantly higher muramidase activity than PMN Monocyte enriched leukocyte preparations (58, 37 and 29%), practically free of lymphocytes, from 3 subjects with reactive monocytosis, yielded enzyme concentrations in the upper normal range for PMN

Figure 3 shows the importance of rapid separation of the cellular elements from the whole blood These experiments were prepared with anticoagulated or clotted blood, stored at various temperatures after venipuncture, in which the serum (or plasma) was not separated from the coagulum or RBC sediment for prolonged time intervals It can be seen, that a significant temperature-dependent increase of muramidase activity occurs in the supernatant serum after 4–5 hours at 22 and 37°C, and after 8–10 hours at 4°C, presumptively as a result of cellular disintegration

Muramidase activity of inflammatory cells The muramidase activity observed in exudate leukocytes at various stages of artifically induced, quantitatively measured inflammatory reactions of 12 human volunteers is shown in figure 4 Since 95–100% of all exudate cells were PMN, their enzyme activity could be compared with the muramidase content of isolated, circulating PMN of the same individuals In all the exudate WBC collected within 2–8 hours of their emigration from the blood stream the muramidase content was not significantly different from the value for blood PMN, although a slight increase was observed at 2 4 and 6 hours after skin abrasion A marked decrease of muramidase activity to 2 6 µg/10^6 (median value) was seen, however, in exudate PMN [illegible]

ut [illegible]

nt [illegible]

p[illegible]ologically [13], but the Trypan Blue exclusion test indicated, that even in 24 hour exudates more than 90% of all PMN were unstained and viable by this common test [20]

Extracellular muramidase of serum and inflammatory exudates Figure 5 summarizes median and range of muramidase activity observed in the initial chamber serum and in subsequent cell free inflammatory exudates into aliquots of this same serum 2 24 hours following skin abrasion The median muramidase activity for 52 normal sera was 18 µg/ml with a mean value (including 2 standard deviations) of

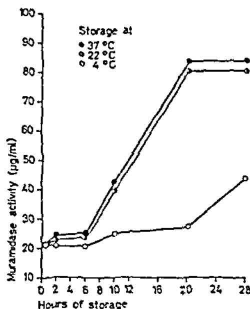


Fig. 3. Influence of duration and temperature of storage on muramidase activity of supernatant serum in clotted whole blood.

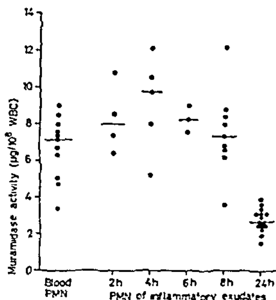


Fig. 4. Muramidase activity (median and range) of PMN suspension from blood and inflammatory exudates, at various stages of the exudative process. (All samples contained 95% PMN.)

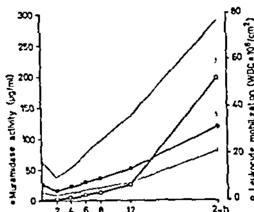


Fig 5. Extracellular muramidase activity of human inflammatory exudates during study of localized leucocyte mobilization (LLM) ● Median noncumulative muramidase concentrations, screen = correspond ng ranges of muramidase activity O median non-cumulative LLM during various collection intervals.

18 7±6 3 µg/ml The respective values for enzyme activity in heparinized plasma from 18 of these same subjects measured 12 6 µg/ml and 14 1±4 9 µg/ml

After an unexplained initial decrease of muramidase activity in the 2-hour exudate, the enzyme level gradually rose to the initial value during the subsequent 2 hour exudates up to 6 hours Significant elevations of median enzyme activity to 34 6 and 50 4 µg/ml were observed during the 6–8- and 8–12 hour intervals ($p<0\,05$ and 0 01 [illegible]

exudate supernatants was roughly proportional to the observed leukocyte mobilization

If PMN-rich 24-hour exudates were allowed to stand and disintegrate at room temperature for another 24 to 48 hours, muramidase concentrations of 400–900 µg/ml could be demonstrated repeatedly

Enzyme recovery Based on a median LLM of 53×10⁶/cm² mobilized exudate cells (95–100% PMN) in the 24-hour chamber specimens and a median muramidase content of 7 2 µg per 10⁶ blood PMN, the ex-

pected enzyme activity of this exudate cell mass would amount to 381.6 μg. Theoretically, this value should match the observed median extracellular and intracellular muramidase activity in the 2 ml exudates at 24 hours, from which value the inherent muramidase content of the native chamber serum should be substracted (2.6 μg×53)+(112 μg/ml−22 μg/ml)×2 ml = 317.8 μg. The median recovered enzyme activity thus falls short of the expected value by 63.8 μg or 17% of the expected value, which corresponds to an enzyme recovery of 83% in the 24-hour samples.

Bacterial studies. All final chamber exudates as well as selected intermediate specimens were cultured for possible bacterial contamination. With the usual standards of sterility observed in filling and emptying the skin chambers, contaminations occurred in less than 2% of all leukocyte mobilization experiments, and these tests were analyzed separately. In 2 deliberately contaminated chambers in healthy physician volunteers, autologous non-hemolytic staphylococci were cultured from both 24-hour chamber exudates, despite extracellular muramidase concentrations of 290 and 320 μg/ml respectively in the exudate supernatant. The size of the bacterial inoculum, transmitted into the chamber system from the individual's own non-disinfected skin, was uncontrolled in these tests, however.

Discussion

The data presented herein as well as the studies of JENSEN *et al.* [14] in rabbits clearly indicate a significant increase of the weakly bacteriolytic enzyme muramidase (lysozyme) at inflammatory sites. It is now generally accepted that among the cells of the hematopoietic system, muramidase is located only in granulocytes and their maturing precursors, as well as in monocytes [3, 4, 10–12]. Results obtained from sufficiently purified populations of peripheral blood cells and exudate cells in the present study confirm this finding. Normal human granulocytes contain approximately 7 μg of muramidase per million cells (based on an egg-white lysozyme standard), a value well comparable to the 6.2 μg/10^6 for 'non-lymphocytic' blood leukocytes published recently by NOBLE and FUDENBERG [20]. These workers employed a different (turbidimetric) enzyme determination method [22] and performed their assays in mixed leukocyte suspensions, partially cor-

rected for muramidase free lymphocytes PERILLIE *et al* [4], using the same turbidimetric method as well as later on the lysoplate assay [3], have reported values of 0.5–3 μg per 10^6 mixed normal leukocytes (mean = 1 μg approximately), a value lower than all other authors (table I)

Even though our own data are rudimentary, monocytes on an iso-cellular basis contain approximately the same amount of intracellular muramidase as do PMN This situation may be entirely different in hematologic diseases, especially monocytic and myelo-monocytic leukemia, in which increased serum muramidase levels and especially excessive muramidasuria were reported out by OSSERMANN *et al* [3, 27] Due to the small absolute number of circulating monocytes in health, these cells probably do not contribute significantly to the serum or plasma muramidase levels physiologically, as mentioned by FINCH *et al* [22]

Table I Survey of quantitative muramidase content of normal human blood leucocytes

Cell population	Muramidase activity in $\mu g/10^6$ leucocytes			
	FLANAGAN *et al* [12]	PERILLIE *et al* [4]	NOBLE and FUDENBERG [21]	Present study
	1955	1968	1967	1968/69
Mixed leukocytes	6.5[1]	1.0[1]	5.0[2]	4.0[3]
Non lymphocytic WBC			(6.2)[4]	(5.8)[4]
Granulocytes		–	–	7.2
Monocytes	–	–	–	8.3
Lymphocytes	0	–	–	0.4
Erythrocytes	0	–	–	<0.01
Platelets	0	–	–	<0.01
Enzyme assay	turbidimetric	turbidimetric	turbidimetric	lysoplate
Enzyme standard	egg-white	egg-white	egg-white	egg-white
Cell lysis	F+T	F+T[S]	sonication	F+T

[1] PMN content unknown
[2] Median PMN = 81%, monocytes = 0% (dextran sedimentation)
[3] Median PMN = 68%, monocytes = 2% (gravity sedimentation)
[4] Values calculated on basis of WBC differential smear
F+T = Rapid freezing and thawing cycles, S = with additional 0.1 N hydrochloride extraction.

The study of controlled inflammatory reactions within a closed chamber system, yielding nearly pure granulocyte exudates, clearly indicates that PMN are the main source of muramidase in 2–24-hour inflammatory exudates. The longer that migrating cells stay in the exudate serum, intracellular muramidase decreases and extracellular enzyme levels increase. Large exudate macrophages, most probably derived from circulating monocytes [23, 24] do not seem to contribute significantly to the muramidase increase at inflammatory sites during the early phase of acute inflammation, but may be of increasing importance in later stages.

No definite explanation can be given, why the recovered muramidase activity in the 24-hour exudates falls somewhat short of the theoretical expected value. Incomplete enzyme extraction from leukocytes might be the cause. If significant numbers of exudate cells would have disintegrated during the mobilization process, the opposite finding would be expected.

Since PMN and monocytes contain relatively large quantities of muramidase, careful handling of blood samples for serum and plasma muramidase levels and special attention as to purity of analyzed leukocyte populations is required. We estimate from our results, that possible damage to and enzyme liberation from only 10% of blood granulocytes during the process of clotting and especially centrifugation could increase the serum muramidase level by 3–5 μg/ml. Centrifugation force and time interval between blood sampling and separation of serum or plasma from the cellular elements are of critical importance for meaningful muramidase results. Enzyme levels in 'mixed' leukocyte suspensions will always be a function of the number of contaminating PMN and possibly monocytes. This is of special significance, if 'nearly' pure populations of cells otherwise known to be virtually free of muramidase (lymphocytes, blasts, RBC, etc.) are assayed. It is probably mainly on this basis, that conflicting and non-representative muramidase activities in leukemic leukocytes have been published [4, 20], a subject, which will be dealt with in a separate communication [25].

It is conceivable that the 'decrease' in muramidase activity observed in the early 2- and 4-hour leukocyte chamber specimens represents only correction by diffusion of an artificially increased initial serum muramidase level, resulting from PMN and monocyte breakdown during clotting and preparation of the serum. This is also suggested

by the fact that heparinized plasma yielded usually muramidase concentrations 30–50% lower than serum of the same donor, although the possibility of interference of heparin with the enzyme assay [3] has not been fully excluded. Another explanation for this strange early extracellular enzyme decrease, seen in nearly all LLM-experiments, could involve possible enzyme inactivation by local factors resulting from the fresh skin abrasion, such as postulated for kidney cell homogenates [26]. Yet another hypothetical alternative would involve possible enzyme uptake by early migrating PMN.

The practical clinical implications of the observed significant increase of muramidase activity at inflammatory sites cannot be fully elucidated at present time. JENSEN *et al.* [14] emphasised the potential significance of high concentrations of extracellular muramidase. Levels of 500–1000 µg/ml were obtained from our cell rich 24-hour chamber exudates containing $40-100 \times 10^6$ PMN, if these cell suspensions were either subjected to lysis or simply left to stay and disintegrate. Regarding the rather large exudate volume of 2 ml serum in our skin chamber study, even much higher local muramidase concentrations may occur at inflammatory sites in the tissues. Preliminary bacteriologic studies with rather large and non-controlled inocula, however, showed no convincing inhibition of the establishment of staphylococcal auto-infection. More discriminatory studies with controlled inocula and various organisms are needed. The skin chamber approach lends itself ideally to such investigations since it permits simultaneous observation of leukocyte exudation, bacterial phagocytosis and bactericidal enzyme concentrations. Studies of the influence of these factors on bacterial survival are currently in progress.

Summary

Determinations of extracellular and intracellular muramidase (lysozyme) activity were performed in serum, plasma, blood cells and artificially induced sterile inflammatory exudates of 57 healthy subjects. Granulocytes and monocytes had similarly high muramidase levels (7.2 and 8.4 µg/10^6). Lymphocytes contained only trace amounts (0.4 µg/10^6) and platelets and RBC no measurable enzyme activity. Intracellular muramidase levels in exudate PMN decreased markedly in the 24-hour chamber exudates. Conversely, the extracellular enzyme concentrations increased significantly in the 8-, 17- and 24-hour exudates, roughly proportional to the extent of cellular emigration. Very high muramidase concentrations were observed in PMN-rich exudates after cellular lysis. Preliminary bacterial inoculation studies revealed establishment of staphylococcal autoinfection despite of high muramidase activity in the exudate serum.

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white blood cells is about 100–150 times higher than in red cells Therefore, the quantity of red blood cells in leucocyte samples is too small to influence the practical error of the determination Usually the control (co) preparation of red cells did not reveal any TKA at all Sometimes the activity was a badly measurable fraction of one unit, which had no practical significance for the final result of the determination procedure It seems obvious that this control (co) preparation of red cells, which serves no useful purpose, could be totally left out of the series

The result of TKA determination of a 'normal leucocyte population is to be considered as a sum of many factors The leucocyte population *in vivo* consists of many kinds, the mutual quantity and also the degree of maturity of which varies a great deal The same kind of cell can be found in different phases [34] The fact, that leucocytes are uniquely sensitive to mechanical or chemical damage in the isolation procedure [2], is one of the well known methodical difficulties in the TKA determination of leucocytes

In consideration of these methodical points of view it is important before further investigations to collect sufficient material about 'normal' TKA-variation of leucocytes By using the present method it seems obvious that the common TKA of leucocytes of a test person s blood over a shorter period (e g at intervals of hours or some days) remains almost equal, on the presumption that there are no pathological factors (e g a strong infection or allergic reaction) between the samplings, which affects the test person The TKA determinations of isolated leucocyte types could produce important additional knowledge of the function of this enzyme in the leucocyte population of blood This being the case the diverging TKA results (table III) in this investigation do not so far merit further discussion

Summary

Transketolase activity (TKA)determinations have been made in white blood cells isolated from peripheral venous blood of 52 persons 41 of whom were controls The TKA

... women) was 7 7±1 6 units

Very low TKA

kemias aplastic

nation of white

blood cells and the reliability of the results are discussed

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Autoradiographische und zytophotometrische Untersuchungen zur Proliferation erythropoetischer Vorstufen im Knochenmark unreifzelliger Leukämien[1]

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Mit verschiedenen Methoden wurde eine verminderte Proliferation pathologischer Blasten unreifzelliger Leukämien [Übersicht bei 13] und der Erythroblasten beim Morbus Di Guglielmo [8] nachgewiesen. Ebenso wurde gezeigt, dass diese Erkrankungen nicht auf eine Zellart beschränkt sind und als Panmyelosen verlaufen können [10, 28]. Wir untersuchten daher, ob in Parallele zu den bekannten Proliferationsstörungen der weissen Reihe auch solche in den roten Vorstufen nachweisbar sind.

Material und Methodik

Insgesamt wurden 9 Patienten mit unreifzelligen Leukämien und 6 hämatologisch gesunde Personen untersucht. Bei den Leukämiepatienten handelte es sich um 7 unbehandelte Fälle und um 2 Patienten in einem Remissionsstadium nach zytostatischer Therapie (Tab. 1).

Autoradiographie und Markierung. Markblut wurde durch Sternalpunktion und Aspiration mit heparinisierten Spritzen gewonnen, im Verhältnis 1:1 mit Nährmedium TC 199 (Burroughs Wellcome, London) verdünnt und in Suspensionskulturen angesetzt. Nach Zusatz von ^{3}H-Thymidin (The Radiochemical Center Amersham, spez. Aktivität 5000 mC/mM; 1 µC/ml Zellkultur) wurde 1 Stunde bei 37 °C in einem Wasserbad mit automatischem Schüttler inkubiert. Anschliessend wurden von den Markbröckeln Quetschpräparate angefertigt, luftgetrocknet und in Alkohol fixiert.

[1] Die vorliegenden Untersuchungen wurden mit Unterstützung des Fonds »Kampf dem Krebs« durchgeführt.

Tabelle I Hämatologische Daten von 9 Patienten mit unreifzelligen Leukämien

	De 32a ♂	Ma 70a♂	Fr 66a ♂	Ra 82a ♂
Diagnose	Erythro-leukämie	Myelobl.-leukämie	Promyeloz.-leukämie	Promyeloz.-leukämie
Ery/mm³ × 10⁶	2,3	1,8	3,0	3,8
Hb, g%	7,6	5,7	8,9	6,0
Reti, ‰	4	28	35	7
Leuko/mm³ × 10³	5,2	2,3	48	5
Thrombo/mm³ × 10³	50	5	10	37
Blut				
Leukäm. Blasten, %	3	9	25	68
Erythroblasten, %	13	10	4	5
Knochenmark				
Granulo/Erythropoese	1,2	0,88	3,75	4,85
Morph. d. Erythrobl.:				
Megaloblasten	+++	++	0	0
Kernabsprengungen	++	+	0	0
Doppelkernige	++	++	0	0

[1] In Remission.

Autoradiographie Mit Ilford G5 Emulsion wurden Dipping-Autoradiogramme angefertigt, 1 Woche bei 4°C exponiert, mit Ilford ID 19 entwickelt, fixiert und bei pH 5,75 nach Giemsa gefärbt. Backgroundkorrekturen wurden mit Hilfe von nicht markierten, [illegible] Vergleich [illegible]

[illegible] Schichtdicke fast konstant 0,7 µm betrug Dies wurde durch Messung der Schichtdicke mittels Vielstrahl-Interferenzmethode nach TOLANSKY geprüft [27] Die ³H-Thymidin-[illegible] 500 erythropoetischen und granulopoetischen Vorstufen [illegible] ausgezählt.

Mitoseindex Zur Bestimmung der Mitoseindices der Erythroblasten wurden Knochenmarkausstriche verwendet, die unmittelbar nach der Markaspiration gewonnen wurden. Neben 7 Leukämiepatienten wurden 3 Kontrollpersonen ausgewertet und jeweils 3000 bis 5000 teilungsfähige Erythroblasten (E_1–E_4) ausgezählt. Um Unsicherheiten bei der Erkennung von Prophasen zu vermeiden, wurden nur Meta-, Ana- und Telophasen ausgewertet.

[illegible] in Pro- [illegible] ortho- [illegible] senkrecht zueinander stehenden Durchmessern bei 6 Leukämiepatienten und 6 Normalpersonen bestimmt

Tabelle I (continued)

Ro 71a ♂	Ca 28a ♂	Ba 73a ♂	Ke 22a ♂	Ne 26a ♀
Myelobl.- leukämie	Promyeloz.- leukämie	Promyeloz.- leukämie	Myelobl.- leukämie[1]	Myelobl.- leukämie[1]
3,6	3,3	2,9	3,7	3,7
10,6	10,6	8,9	10,6	12,0
45	4	9	26	9
6,7	4,7	13	3,7	4
184	9	11	193	370
0,5	75	7	0	0
0	1	0,5	0	0
0,85	15	15	6,01	2,63
0	0	0	+	+
0	+	0	0	0
0	0	0	0	+

Kombinierte Zytophotometrie und Autoradiographie. Knochenmarkzellen wurden wie oben in vitro markiert, ausgestrichen und nach Pappenheim gefärbt. Geeignete Präparatstellen wurden bei mittlerer Vergrösserung photographiert, um die Ergebnisse aufeinanderfolgender Untersuchungen (morphologische Differenzierung, Kerngrösse, relativer DNS-Gehalt und 3H-Thymidininkorporation) an der Einzelzelle miteinander korrelieren zu können. Nach Eintragung der morphologischen Differenzierung und Kerngrösse in die Photographie wurden die Präparate gewässert, mit absolutem Methanol/Äthanol (1:1) entfärbt und anschliessend nach Feulgen gefärbt. Dabei wurde die saure Hydrolyse mit n HCl bei 60°C ausgeführt, jedoch auf 7 min reduziert, um Proportionalitätsfehler der Feulgen-Färbung [3] zu vermeiden und den Markierungsverlust gering zu halten. Bei 4 unbehandelten Stammzell-Leukämien und 3 Kontrollpersonen wurde an je 150–300 Erythroblasten mit einem integrierenden Mikrodensitometer (GN 2, Fa. Barr & Stroud, Glasgow) nach DEELEY [7] zytophotometrisch der relative DNS-Gehalt bei einer Wellenlänge von 560 nm bestimmt. Anschliessend wurden die Präparate wie oben autoradiographiert.

Zytochemische Differenzierung der unreifzelligen Leukämien. Für die Zelldifferenzierung der untersuchten akuten Leukämien wurden folgende zytochemische Verfahren herangezogen [1]: PAS-Reaktion [18], Berliner-Blau-Reaktion [18], Sudanschwarz-B-Färbung [26] sowie die Enzymnachweise für saure Phosphatase [2], Peroxydase [23], α-Naphthyl- und Naphthol-AS-LC-Acetat-Esterase [15, 24]. Die Kernfärbungen wurden je nach der Farbe des zytochemischen Reaktionsproduktes mit saurem Hämalaun nach Mayer, mit Kernechtrot oder mit der Feulgenreaktion durchgeführt (Tab. II).

Tabelle II Zytochemische Befunde von 7 Patienten mit unbehandelten unreifzelligen Leukämien

Diagnose	PAS	Fe	Saure Phosphatase
Myeloblastenleukämie Ma 70a ♂	0 – ±	0	0 – +
Promyelozytenleukämie Fr 66a ♂	+	0	+ – ++
Promyelozytenleukämie Ra 82a ♂	+	0	+
Promyelozytenleukämie Ca 28a ♂	+ – ++	0	+ – ++
Promyelozytenleukämie Ba 73a ♂	++ – +++	0	++ – +++
Myeloblastenleukämie Ro 71a ♂	0 – +	0	+
Erythroleukämie De 32a ♂	Erythroblasten 0 – ++	Erythroblasten 0 – ++	Erythroblasten + – ++

Ergebnisse

^{3}H-Thymidin-Markierungsindex Der Markierungsindex der Erythroblasten lag bei unbehandelten Stammzell-Leukämien signifikant unter den Werten der Kontrollgruppe ($P < 0{,}001$), während sich die Indices der beiden Patienten in Remission von letzterer nicht unterschieden Der Index betrug bei der Kontrollgruppe $31 \pm 0{,}7\%$, während er bei Leukämikern auf $11{,}3 \pm 2{,}2\%$ herabgesetzt war Auch der Markierungsindex der granulopoetischen Vorstufen bei unbehandelten Leukosen war deutlich vermindert, und zwar ebenfalls auf etwa ein Drittel der Kontrollgruppe (6,1 gegenuber 19,6%) Die für die ganze Gruppe im Mittel ähnliche Verminderung der Markierungsindices der roten und weissen Vorstufen war im Einzelfall nicht immer nachweisbar Silberkornzählungen wurden lediglich an polychromatischen Erythroblasten durchgeführt und ergaben bei Leukämikern und der Kontrollgruppe vergleichbare Werte (Tab III) Bei 2 Patienten (Ra, De) wurde auch der Markierungsindex der Erythroblasten im peripheren Blut ausgewertet Er betrug jeweils etwa die Halfte des Index im Knochenmark (3,8% und 15,9% gegenüber 7,9% und 23,0%)

Tabelle II (continued)

α-N*	Peroxydase	Sudan	Naphthol-AS-LC-Acetat Esterase
+	0 – +	0 – +	+
+	+ – +++	++ – +++	+ – ++
+	+ – ++	+ – ++	+
+	+++ – ++++	+++ – ++++	++ – ++++
+	0 – +++	0 – +++	+ – ++
+	0 – +	0 – +	+
Erythroblasten ++	Myeloblasten +	Myeloblasten +	Myeloblasten +

* α Naphthyl-Esterase

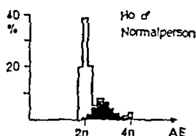
Tabelle III ^{3}H Thymidin Markierungsindex der erythro- und granulopoetischen Vorstufen im Knochenmark

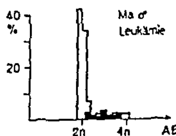
	^{3}H Thymidin-Markierungsindex (%) (E^1–E^3)	^{3}H Thymidin-Markierungsindex (%) (Myelobl. – Metamyc.)	Mittlere Silberkornzahl (E^4)
Normal n = 6	31,1 ± 0,74	19,6 ± 1,2	52 ± 10,5
Leukämien n = 7	11,5 ± 2,17	6,1 ± 2,0	60 ± 3,5
Leukämien in Remission	35		–
n = 2	33	–	–

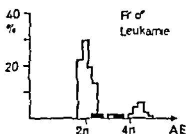
Mitoseindex. Der Mitoseindex der Erythroblasten von unbehandelten Leukämiepatienten lag ebenfalls signifikant unter den Vergleichswerten bei hämatologisch Gesunden ($P < 0,01$). So betrug der Index bei diesen Leukämiepatienten 17,2‰ gegenüber 32,0‰ der Kontrollgruppe. Die 2 Leukämiker in Remission unterschieden sich nicht von den Normalpersonen. Wurde die Zahl der erythroblastischen Synthe-

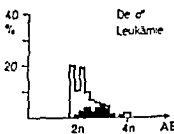
Tabelle IV. Mitoseindex teilungsfähiger Erythroblasten im Verhältnis zur ³H-Thymidin-Markierung

	Mitoseindex (‰)	³H-Thymidin-Markierungsindex: Mitoseindex
Normal n = 3	32 ± 0,58	14,6 ± 0,62
Leukämien n = 5	17,2 ± 2,63	10,2 ± 0,71
Leukämien in Remission	*30*	*16,6*
n = 2	36	16,1









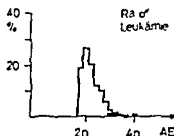


Abb. 1. DNS-Verteilungskurve der Knochenmarkerythroblasten von 4 Patienten mit unreifzelligen Leukämien und einer Normalperson. Schwarze Felder: ³H-Thymidineinbau.

sezellen zur Zahl der Mitosezellen ins Verhältnis gesetzt, so war dieser Quotient bei den Leukämien gegenuber der Kontrollgruppe signifikant vermindert (P <0,01) (Tab IV) Zwischen dem Schweregrad der Anämie und der Verminderung der Mitose- und Markierungsindices bestand keine engere Beziehung

Kombinierte Autoradiographie und Zytophotometrie Die Kombination von Autoradiographie und Zytophotometrie ermöglicht eine Auftrennung von unmarkiert gebliebenen Zellen in die G_1- beziehungsweise G_0- und G_2-Phase Bei den untersuchten Leukämikern kommt eine Verminderung der Zellen in DNS-Synthese zugunsten von G_1- beziehungsweise G_0-Zellen deutlich zum Ausdruck, nur eine der drei in dieser Serie ausgewerteten Normalpersonen wurde in die Abbildung aufgenommen (Abb 1) Bei einem Patienten (Fr) ergaben sich An-

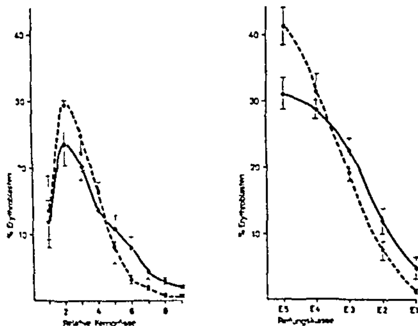


Abb 2 Verteilung der Kerngrößen und Reifungsklassen der Knochenmarkerythroblasten von 6 Patienten mit unreifzelligen Leukämien (ausgezogene Linie) und 6 Normalpersonen (unterbrochene Linie)

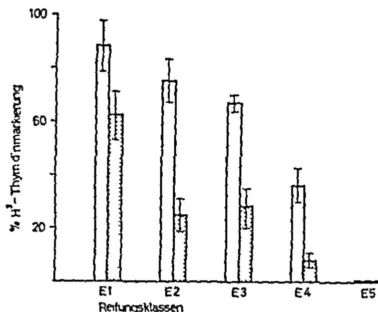


Abb 3 Durchschnittlicher Kompartmentmarkierungsindex der Knochenmarkerythropoese bei 5 Patienten mit unreifzelligen Leukämien (dunkle Säulen) und 5 Normalpersonen

haltspunkte für das Vorliegen eines zweiten, ungefähr tetraploiden Erythroblastenstammes, bei einem weiteren Patienten (De) war der Gipfel der präsynthetischen Zellpopulation auffallend breit und anscheinend etwas nach links verschoben

Kerngrossenbestimmung, morphologische Differenzierung und relative Kompartmentmarkierung Die morphologische Differenzierung und die Kerngrössenbestimmung an Erythroblasten von unbehandelten Leukämiepatienten ergaben übereinstimmend eine im Vergleich zur Kontrollgruppe signifikante Verminderung kleiner zugunsten grosserer Erythroblasten ($\chi^2 = 34{,}997$, Fg 8, P <0,001) (Abb 2) Wurden die relativen Kompartment-Markierungsindices verglichen, so waren diese nur in den mittleren Reifungsklassen (E_2–E_4) signifikant (P < 0,005) gegenüber der Norm vermindert (Abb 3)

Diskussion

Mit verschiedenen, methodisch voneinander unabhängigen Untersuchungen erhielten wir Hinweise auf eine im Durchschnitt verminder-

te Teilungsfähigkeit von Erythroblasten bei unreifzelligen Leukämien Bei der Berechnung des ^{3}H-Thymidin-Markierungsindex dieser Zellen betrugen die Werte nur ein Drittel der Kontrollgruppe An den Erythroblasten der letzteren fanden wir im Mittel 31 % ^{3}H-Thymidin-inkorporierende Zellen, ein Ergebnis, das mit den von anderen Autoren auf autoradiographischem [25] und zytophotometrischem Weg [17] erhaltenen Werten gut übereinstimmt. Die Erniedrigung der Markierungsindices war im Durchschnitt für Erythroblasten und die weissen Vorstufen ähnlich Eine Verlängerung der durchschnittlichen Generationszeit der leukämischen Blasten auf etwa das Dreifache wurde mehrfach beobachtet [Übersicht bei 13] Bei Vergleich von *in vitro*-Ergebnissen mit Befunden nach ^{3}H-Thymidin-Markierung *in vivo* wurde zumindest für die weisse Reihe ausgeschlossen, dass diese Unterschiede durch Artefakte unter den *in vitro*-Bedingungen verursacht sind [22]

Auch bei der Auswertung der Mitoseindices roter Vorstufen ergaben sich in der Gruppe der Leukämiker Hinweise auf eine verminderte Proliferationsaktivität Der Index war im Mittel auf fast die Hälfte der Kontrollgruppe herabgesetzt Zellen, die sich in der Prophase befanden, liessen sich nicht immer sicher von solchen der Interphase abgrenzen, so dass bei der Auswertung lediglich Zellen in den späteren, morphologisch gut abgrenzbaren Phasen der Teilung berucksichtigt wurden Dadurch lag der Mittelwert der Kontrollgruppe mit 32‰ niedriger als der anderer Autoren, die Prophasenzellen mit einbezogen hatten und Werte um 44‰ erhielten [6, 20] (Der Anteil der Prophase beträgt etwa ein Fünftel der Mitosezeit [21]) Unter Berücksichtigung dieses [illegible]
ein In[illegible]
rungs[illegible]
Stammzell Leukämien war ausgeprägter als die des Mitoseindex Schlechte Kulturbedingungen als Ursache für das unterschiedliche Verhalten sind, wie vorher diskutiert, eher unwahrscheinlich Es scheint naheliegender, an eine im Durchschnitt verlängerte Mitosedauer der erythropoetischen Vorstufen im Mark der Leukämiker zu denken Eine verlängerte Mitosedauer leukämischer Blasten wurde direkt beobachtet [4] und auch indirekt errechnet [13]

Auch zytophotometrisch war der Zellanteil mit einem der S-Phase entsprechenden DNS-Gehalt bei allen Patienten mit unbehandelten Stammzell Leukämien gegenüber der Norm [17] vermindert Dieser

Befund steht in Übereinstimmung mit den kürzlich von MÜLLER mitgeteilten Beobachtungen [16]. Bei einem Patienten fanden sich Anhaltspunkte für das Vorliegen eines zweiten, ungefähr tetraploiden Erythroblastenstammes. Ähnliche Beobachtungen wurden auch an unreifen, weissen Zellen gemacht [9]. Bei einem weiteren Patienten war der Gipfel der präsynthetischen Zellpopulation auffallend breit und der 2n Gipfel etwas nach links, wohl im Sinn eines niedrigeren DNS Gehaltes, verschoben. Die Zellen mit einem DNS Gehalt unter 2n wurden morphologisch fast ausnahmslos als orthochromatische Erythroblasten charakterisiert. Dieser Befund könnte durch verzögerte Kernausstossung bei gestörter Zellreifung erklärt werden.

Kerngrossenmessung und morphologische Differenzierung an den Erythroblasten der Leukämiepatienten zeigten übereinstimmend ein relatives Überwiegen grösserer, junger Formen. Die Ursache dieser «Reifungsstörung» ist nicht geklärt, und es stellt sich die Frage, wieweit die beobachtete Proliferationsstörung dazu in Beziehung steht. An den weissen Vorstufen geht die Verminderung von Blasten in der Zellteilung mit einer gestörten Bildung funktionsfähiger, reifer Zellen einher. Trotz eines vergleichbaren Defizits DNS synthetisierender roter Vorstufen war diese «Reifungsstörung» vergleichsweise weniger ausgeprägt.

Der relative Kompartment-Markierungsindex lag nur in den mittleren Reifungsklassen signifikant unter den Kontrollen, deren Werte mit den Ergebnissen anderer Autoren nach Markierung *in vitro* [25] und *in vivo* [5] gut übereinstimmen. Ebenso fanden sich bei unreifzelligen Leukämien nach Markierung *in vivo* [14] und *in vitro* [11] deutliche Hinweise für eine herabgesetzte bis aufgehobene Teilungsfähigkeit der mittelgrossen und kleinen pathologischen Blasten.

Nur bei einem unserer Patienten war das Vollbild einer Erythroleukämie gegeben (De). Bei einem weiteren Patienten (Ma) fanden sich neben einer absoluten Vermehrung der roten Vorstufen im Knochenmark auch vermehrt doppelkernige Zellen und an Megaloblasten erinnernde Formen. Bei diesen Patienten war die relative Verminderung DNS synthetisierender Erythroblasten nicht ausgeprägter als bei den Fällen mit weniger auffälligen morphologischen und zytochemischen Anomalien.

Unsere Befunde scheinen für das Vorliegen einer Proliferations- und wahrscheinlich auch Reifestörung der roten Vorstufen bei unreifzelligen Leukosen zu sprechen. Sie bestätigen damit quantitativ die zytogene-

tischen Untersuchungen, die auf eine Einbeziehung der Erythropoese in die leukämische Entartung hinweisen [10, 28]. Es liegt nahe, in der Proliferations- und Reifungsstörung einen Teilfaktor beim Zustandekommen der Anämie zu sehen, die sicher keiner reinen Verdrängung entspricht, sondern wohl häufig komplexer Natur ist [19, 29].

Frau Ursula Michelmayr sind wir für die gewissenhafte technische Mitarbeit zu grossem Dank verpflichtet. Weiters möchten wir Dr. D. Abramann vom Institut für Physikalische Chemie für die Schichtdickenbestimmung unserer Dipping-Filme danken.

Zusammenfassung

An 9 Patienten mit unreifzelligen Leukämien wurden autoradiographische und zytophotometrische Untersuchungen sowie Bestimmungen der Mitoseindices und der Kerngrössenverteilung an Erythroblasten vorgenommen und die Ergebnisse mit Normalpersonen verglichen. Die Anzahl von Erythroblasten in DNS-Synthese bzw. beziehungsweise in Zellteilung war bei unbehandelten Leukämien gegenüber der Kontrollgruppe signifikant erniedrigt. Dies wurde als Ausdruck einer in diesen Zellen im Durchschnitt verlängerten Generationszeit angesehen. Die Verlängerung der durchschnittlichen Generationszeit war der der leukämischen Blasten vergleichbar. Zwei Patienten in einem Remissionsstadium unterschieden sich nicht von den Kontrollen. Die Befunde könnten als Hinweis für eine Einbeziehung der Erythropoese in die leukämische Proliferationsstörung gewertet werden.

Summary

On 9 patients with stem cell leukemias we investigated some aspects of red cell proliferation employing ³H-thymidine incorporation, microphotometry, mitotic indices and measurements of the nuclear diameter. The number of erythroblasts in DNA-synthesis as well as in mitosis was significantly reduced in comparison with normal subjects. The results were in agreement with a prolonged average generation time of erythroblasts and comparable to findings on leukemic blast cells. In two patients in remission those parameters were within the normal range.

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A New Abnormality of Platelet Function

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At present 3 lifelong bleeding disorders are recognised, attributable at least in part to abnormalities of platelet function Common to all three are a prolonged bleeding time and decreased platelet adhesiveness, by the method of Salzman [17] In the first, von Willebrand s disease, no other platelet functional abnormality can be detected at present In the second, thrombocytasthenia (thrombasthenia, Glanzmann's disease), there is impaired clot retraction, and aggregation of platelets cannot be initiated *in vitro* by ADP or adrenaline and it is grossly impaired by connective tissue extract (CTE) and thrombin [3, 6, 20] The third, known as thrombopathia [19], 'new platelet abnormality' [3, 6, 8] or Portsmouth syndrome [16], is characterised by an atypical pattern of platelet aggregation with CTE *in vitro*, a reduced or absent release of ADP from platelets, and occasionally, an abnormal platelet factor 3 availability (PF3a) We describe here a new abnormality of platelet function associated with a lifelong bleeding tendency, prolonged bleeding time, reduced platelet adhesiveness and abnormal platelet aggregation

Case Report

E.M S is a 72 year-old female first seen aged 58 Spontaneous bruising has been present since early childhood and she experiences frequent and prolonged epistaxes Over the 15-year period she has been under observation there have been 3 episodes of melaena once necessitating massive blood transfusion During the reproductive period menorrhagia was always present. Although she retains most of her second dentition the extraction of teeth on two occasions was complicated by bleeding for more than 24 h eventually requiring suturing Her most recent episode was an acute haemarthrosis affecting the right knee

She has no living relatives and was always warned that childbearing might be dangerous. She recalls that her father bled excessively from superficial cuts and eventually died from a cerebrovascular accident aged 63. Her only sister died aged 53 from the same cause. Her grandmother on her father's side also died from haemorrhage following childbirth.

Methods

Since techniques are not fully standardized, a brief description of our methods is given. *Platelet adhesiveness* was measured by a slightly modified SALZMAN technique. Blood was obtained by cuffed venepuncture using a 20G disposable siliconised needle [Becton Dickinson (B.D.) 20G, 1½"] and allowed to flow through the filter into a 7 ml vacutainer (B.D. 4739 vacutainer holder B.D. 4893) with standard negative pressure. The filter is constructed [illegible]

[illegible]

before the cuff was released and 45 sec altogether. Three to 5 ml of blood are obtained in this time. If, for any reason, less than 3.0 ml of blood are collected, the result may be unreliably high. The patient's haematocrit must be greater than 35%, otherwise unreliably low results are obtained. Platelet counting was done visually by the phase contrast technique. Our normal range has proved to be 32–65% adhesiveness.

Experimental platelet adhesiveness to collagen was investigated as follows. On a glass microscope slide one drop of CTF is mixed with one drop of citrated platelet rich plasma (PRP), placed beneath a cover glass and observed immediately under the phase contrast microscope. In the normal after a delay of some minutes platelets adhere to the collagen microfibrils and later large aggregates are formed.

Aggregation. Venous blood was collected into a plastic syringe and mixed with 3.8% [illegible]

[illegible]

temperature, 16–20 °C, until testing within 4 h. All the glassware was siliconised.

The platelet aggregation study was performed according to the methods of BORN [1] and O'BRIEN [13]. An EEL model 169 Platelet Aggregation Meter was used linked to a recorder (desk type Servoscribe Potentiometric Recorder, Smith Industries Ltd.). The PRP was divided into 1 ml volumes in disposable polystyrene tubes (45 × 10 mm Luckham Ltd., FT 00441). Each tube was warmed to 37 °C for 5 min in a water bath before testing and transferred into the mixing chamber where it was continuously stirred and maintained at 37 °C (stirrer speed at arbitary number 70). The recorder sensitivity was set at 10 mV, paper speed 600 mm/h. Aliquots of 0.1 ml of the aggregating agents were added to the PRP, aggregation being graphically recorded.

Our aggregating agents were prepared as follows. The sodium salt of ADP (Sigma) was dissolved in barbitone buffer saline (BBS) pH 7.4 at a concentration of 100 µg/ml and stored in 1 ml aliquots at −30 °C (for up to 3 months). Aliquots were thawed once only and ADP was used in final concentration of 0.5 µg/ml and 10.0 µg/ml PRP.

Adrenaline BP was used, prepared freshly each day, diluted in physiological saline to a final concentration of 1.0 µg/ml PRP.

The Sigma preparation of 5-hydroxytryptamine (5-HT) was dissolved in BBS at a concentration of 2.5 mg/ml (a final concentration of 250 μg/ml PPR) and stored in 1 ml aliquots, at 4°C for up to 1 month.

Human connective tissue extract (CTE) was prepared as described by Hovig [9].

Thrombin was prepared from Thrombin Topical (Parke-Davis). Five thousand units were dissolved in 5 ml saline and placed at 4°C. After 4 days aliquots of this solution were further diluted in saline for use at a final concentration of 1 unit/ml PRP. The original solution can be used for up to one month.

ADP release from platelets was measured by the aggregation method, the principle of which is a follows. Aggregation of the platelets under test with CTE [9] or thrombin causes them to release ADP the presence of which in the plasma can be demonstrated by its ability to aggregate normal platelets. In practice, when the aggregation with CTE or thrombin was completed (5 min after the addition of CTE and 3 min after the addition of thrombin) the plasma was centrifuged at 3 500 rpm for 1 min and 0.1 ml or 0.5 ml of the supernatant was added to 1 ml of normal PRP. The amount of ADP released was determined from a standard curve (the concentration of ADP which produces an equivalent aggregation in the same normal PRP).

In addition a method in which ADP release from the test platelets was initiated by kaolin, was used [19].

The PF-3a was determined according to the methods of Hardisty and Hutton [5] and Spaet and Cintron [18] as described by Weiss [19].

Results

Over the 15-year period of observation platelets have been counted on many occasions. Usually the count has been more than 100,000/mm³, but had ranged from 45,000 to 185,000/mm³ (table I). Clinically, two of the most severe bleeding episodes, one of melaena and one haemarthrosis have been associated with platelet counts of 10,000 to 60,000/mm³. During convalescence the platelet count rapidly returned to levels in excess of 100,000/mm³ without specific treatment. Morphologically the platelets appear normal with normal clumping.

Under the phase contrast microscope platelets in PRP adhered normally to connective tissue microfibrils.

Bleeding time (Ivy), Hess' test and platelet adhesiveness were consistently abnormal (table I). Clot retraction was normal at 58% and PF3a was consistently within normal limits.

Platelet aggregation was examined completely on 6 separate occasions over a 6-month period. During this time no medicines of any description were taken. Figure 1c compares a normal aggregation pattern produced by 0.5 μg/ml ADP to that of our patient. The major points of difference to be noted are: (1) the amplitude of the trace remains almost the same in the test PRP compared to a decrease in

Table I. Laboratory results in the patient over the period of observation

Test	Date													
					1967			19[illegible]9			19[illegible]9			
	30.7	5.[illegible]	18.4.[illegible]	23.4.[illegible]	16.[illegible]	20.6	18.7	8.10	11.11	21.11	14.2*	13.[illegible]	15.7	13.8.
Bleeding time (Ivy) normal 0–7 min	30	30		15		$9\frac{1}{2}$	14	11					>15	
Hess test	strongly positive	weakly positive											positive	
Platelet adhesiveness (Salzman %) normal 32–[illegible] %								9	15					14
Platelet count (mm³) normal 1[illegible]0 000–[illegible]00 000	185 000	137 000			48 000	53 000	124 000	1 8 000	67 000	119 000	53 000	145 000	136 000	95 000
Factor VIII assay normal [illegible]0–200 %							80	40						100

* Admitted for melaena
† Admitted for haematemesis [illegible]

amplitude in the normal. Normal platelets under the influence of ADP undergo a change in shape from discoid to 'spiny' spherical [11] indicated graphically in this system by a decrease in amplitude [15]. We interpret our finding to mean that the patient's platelets do not respond normally to ADP with respect to shape; (2) disaggregation, normally appearing within 1 min [14] does not occur.

Figures 1a–d show the abnormal response to ADP again with incremental amounts of from 0.1 to 0.4 μg/ml compared to normal.

Figure 2a shows that the aggregation and change of shape in response to 10 μg/ml ADP is normal. In figure 2b is seen the effect of CTE in the normal and the patient. They differ in that aggregation in the patient is less rapid. The response of the normal and of the patient to thrombin is illustrated in figure 2c. In the normal there is usually a delay of about 10 sec after the addition of thrombin and then the tracing narrows (change in platelet shape) and the aggregation starts and proceeds to completion rapidly, not to be followed by disaggregation. In the patient the tracing narrows and the aggregation starts normally but never becomes complete and disaggregation always follows.

With adrenaline and 5-HT the response is normal (fig. 2d and e). With adrenaline the presence of a second curve [15] is thought to be produced by release of intrinsic ADP which can be seen in patient and normal in figure 2d as a change of slope. Normally with 5-HT one sees weak aggregation followed by disaggregation. Change of shape is always seen.

Incubation of normal PRP with the patient's PPP for 2 h at room temperature gave normal aggregation patterns with ADP and thrombin. Also the addition of normal PPP to the patient PRP did not restore the abnormal aggregation pattern.

ADP-release by kaolin was found to be within the lower normal range, 1.0 μg/ml being present in the platelet poor supernatant plasma. ADP release by CTE was variable. On two occasions it was found to be abnormally low and on a further two within the lower part of the normal range. With thrombin there was no release of ADP. Overall ADP-release is definitely impaired in response to thrombin and probably impaired in response to CTE.

The tests of coagulation carried out when the patient was first seen were normal and remained so over many years when repeated. In particular factor VIII (antihaemophilic factor) levels were normal,

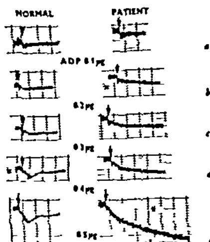


Fig. 1 Aggregation pattern of the patient, produced by incremental amounts of ADP from 0.1 to 0.5 µg ml (final concentration), compared with normal.

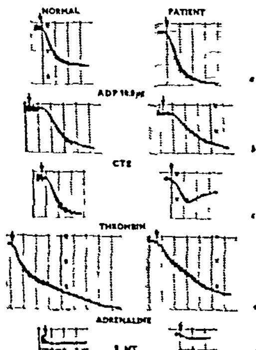


Fig. 2 Aggregation pattern by various aggregating agents in the patient and a normal person.

using the one-stage technique of HARDISTY and MACPHERSON [17]. More recently she has developed a weak non-specific inhibitor which has not affected the severity of the bleeding tendency; her factor VIII level remains normal.

The blood urea is normal (21 mg/100 ml) as are her serum proteins, protein electrophoresis and liver function tests.

With the exception of the transient anaemia following haemorrhage her haemoglobin and white cell count are consistently normal.

Discussion

It is believed that the following is the sequence of events in platelet function during haemostasis. Firstly the platelets adhere to the connective tissue of the damaged vessel wall. The platelets attached to collagen then release ADP under the influence of which further platelets stick to each other and to those attached to the exposed collagen. Thus, a platelet aggregate is formed sufficient to arrest bleeding in a small vessel. Meanwhile the platelet factor 3 becomes available and accelerates coagulation After the completion of the coagulation of the blood, the blood clot is retracted by means of the platelets contained in it.

This sequence of events is investigated in our laboratory by the methods described above. Platelet adherence to collagen is tested by aggregation response to connective tissue extract and by adherence of platelets to collagen microfibrils Release of ADP from platelets is measured by ADP-release methods ADP induced aggregation is assessed by the aggregation method A major abnormality anywhere in the sequence produces a prolonged bleeding time and a decreased platelet adhesiveness

In this patient the bleeding time and platelet adhesiveness are abnormal, strongly suggesting a qualitative platelet abnormality. The Hess test has been positive since the patient first came under observation and was the first abnormality noted. In all the major platelet functional abnormalities thrombasthenia, thrombopathia and von Willebrand's disease, the Hess test is usually positive. The adherence to collagen microfibrils is normal in our patient and so is the aggregation by connective tissue extract (apart from a slight impairment in the speed of aggregation). The ADP-release mechanism

as measured by kaolin, thrombin and connective tissue extract is slightly impaired The aggregation with ADP and thrombin is initiated normally but is abnormal in pattern Mixture experiments, performed using combinations of patient and normal platelet rich and platelet poor plasma, indicate that the defect is located in the patient's platelets and not in the plasma

In vitro, this patient differs from the other platelet functional abnormalities as follows (see also table II)

1 Von Willebrand's *disease* is characterized by normal aggregation and ADP release and low factor VIII in some Our patient shows abnormal aggregation and ADP release and normal factor VIII

2 *Thrombopathia or Portsmouth syndrome or 'new platelet abnormality'* is characterized by incomplete aggregation with connective tissue extract and with adrenaline, normal aggregation with ADP and an occasionally abnormal platelet factor 3 availability In our case the aggregation with adrenaline and the platelet factor 3 availability are normal the aggregation with connective tissue extract is less rapid but proceeds to completion and with small amounts of ADP the aggregation is abnormal

3 *Thrombasthenia* The response to ADP is restricted to change of platelet shape but no aggregation takes place, there is no aggregation with adrenaline and only a poor response to connective tissue extract and thrombin, clot retraction is zero or abnormally low, the platelet factor 3 availability is grossly impaired but ADP release is normal, in the peripheral blood film the platelets do not show normal clumping In our patient the aggregation proceeds normally but is abnormal in pattern with small amounts of ADP (absence of disaggregation and no change in platelet shape) and with thrombin (incomplete aggregation followed by disaggregation) The platelets show normal clumping in the peripheral blood film, clot retraction and platelet factor 3 availability are normal

The aggregation patterns in thrombopathia and thrombasthenia are illustrated in figure 3 and can compared with that of our patient

Despite the obvious *in vitro* abnormalities of platelet function in our patient it is difficult to explain the exact mechanism of the bleeding tendency It seems that the major abnormality is located in the nucleotide mechanisms In a normal person, small amounts of ADP change platelet shape to spherical and produce aggregation followed by disaggregation, change in shape and disaggregation do not occur

Table II Comparison of *in vitro* tests in various platelet functional abnormalities

	Adhesive-ness	BT	Aggregation by				ADP-release	PF3a	Clot retract.	Factor VIII
			ADP	CTE	Thrombin	Adren.				
von Willebrand's disease	ABN	ABN	N	N	N	N	N	N	N	ABN
Thrombopathia, etc.	ABN	ABN	N	ABN	N	ABN	ABN	ABN	N	N
Thrombasthenia	ABN	ABN	ABN	ABN	ABN	ABN	N	ABN	ABN	N
Sprowson anomaly	ABN	ABN	ABN	N	ABN	N	ABN	N	N	N

N normal, *ABN* abnormal.

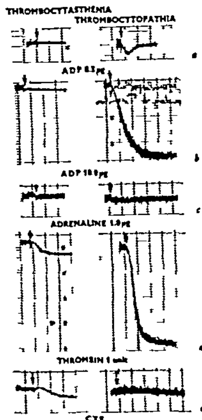


Fig. 3. The aggregation pattern in thrombasthenia (Glanzmann's disease) and thrombopathia (thrombopathia, etc.)

in our subject. The ability of the platelets to change shape and disaggregate are not absent however, both being induced by 5-HT and thrombin. These abnormalities are thus specific to the function of the ADP.

It has been suggested that the disaggregation following aggregation by small amounts of ADP is due to the removal of the ADP by a plasma enzyme system. The rate of disaggregation is correlated to the rate of breakdown of ADP [12]. In this patient, therefore, an absence of such enzymes could explain the absence of disaggregation after small

amounts of ADP. We examined this possibility as follows: 10.0 μg ADP were added to patient's PPP placed at 37°C. At 5-minute intervals 0.1 ml samples of the mixture were added to normal PRP and the induced aggregation observed. Normal ADP decay was demonstrated by progressively weaker aggregation until, after 20 min, no aggregation occurred. This seems to exclude a plasma deficiency of the postulated enzymes.

We believe that disaggregation may be an intrinsic platelet mechanism triggered *in vitro* by aggregation produced by ADP, thrombin and 5-HT (for the two former disaggregation occurs only when small amounts are reacting). In present thinking about platelet function no role has been ascribed to disaggregation *in vivo*. It may be that disaggregation has an important physiological function in the preservation of platelet integrity. Circulating platelets must in certain physiological and disease states be brought under the influence of various stimuli (5-HT, thrombin, ADP and possibly others) to which they can react by aggregation. Disaggregation which follows serves to disperse the platelet clumps and return functionally normal platelets to the circulation. In this patient failure of disaggregation to ADP under stress (haemorrhage) when ADP is released elsewhere in the body may contribute to the evanescent thrombocytopenia by the formation of irreversible platelet clumps.

ADP release from the subject's platelets is also impaired and this might explain the impairment in the speed of aggregation with connective tissue extract. The incidence of a variety of abnormalities concerning the ADP (absence of *disaggregation, no change in platelet* shape and abnormal ADP release) is suggestive of impaired nucleotide metabolism.

Intact ADP production and response appear essential to haemostasis. Here production is moderately abnormal and response very abnormal. Although aggregation occurs, platelet shape does not alter and at a capillary level an aggregate of discoid platelets may produce an ineffective plug.

The abnormal aggregation of the patient's platelets with thrombin is difficult to interpret. We think that the abnormal response of our patient's platelets to thrombin does not contribute to explaining the bleeding tendency. Although thrombin does act on platelets in haemostasis (it causes aggregation, releases ADP and the vasconstrictor amines adrenaline and serotinin from the platelets and makes platelet

factor 3 available), this action can be produced by other substances (ADP and collagen), which are present during haemostasis

The increasing sophistication of the techniques with which we are able to examine platelet function will probably lead to a more detailed and thorough knowledge of the role of platelets in haemostasis and to a better correlation of the *in vitro* findings with the *in vivo* phenomena

We have called this new platelet functional abnormality 'Sprowson Anomaly' after the patient

Acknowledgements We wish to thank the patient for her co-operation in this work, Miss A. Burn for her technical assistance in the blood coagulation study and the Illustration Department of United Manchester Hospitals.

Summary

An unusual case of a lifelong bleeding tendency and family history of bleeding is described. The patient showed a disturbance of platelet function which took the form of an abnormal response to and impaired release of adenosine diphosphate (ADP). To the best of our knowledge this abnormality has not previously been described.

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Akute autoimmunhämolytische Anämie vom Kälteantikörpertyp nach Mykoplasmapneumonie mit tödlichem Ausgang[1]

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Seit den ersten Beobachtungen von Turner *et al.* [29, 30, 31] sowie von Peterson *et al.* [24] ist bekannt, dass es bei «primär atypischen» Pneumonien relativ häufig zu einem vorübergehenden, individuell verschieden starken Anstieg des Kälteagglutinintiters kommen kann. Wahrscheinlich handelte es sich bei diesem Beobachtungsgut um Mykoplasmapneumonien, denn verschiedene Autoren [11, 12, 17, 19, 22] fanden eine signifikante Häufung passagerer Kälteagglutininvermehrungen bei ätiologisch gesicherten Infektionen durch einen Erreger, der zunächst nach seinem Entdecker als «Eaton agent» benannt, von Chanock *et al.* [6, 7] als «Pleuropneumonia like organism (PPLO)» identifiziert und als Mycoplasma pneumoniae bezeichnet wurde.

Nachdem man anfangs vorübergehende Kälteagglutininvermehrungen als spezifisch für diese Krankheitseinheit angesehen hatte, zeigte sich später, dass sie auch bei abakteriellen Pneumonien anderer Ätiologie vorkommen, wenngleich erheblich seltener [9, 19, 22]. Anscheinend führen Mykoplasmainfektionen jedoch bevorzugt zu einer Stimulierung der Kälteantikörperbildung.

Passagere para- bzw. postpneumonische Kälteagglutininvermehrungen haben «reaktiven» Charakter. Sie pflegen mit Heilung der Grundkrankheit spontan abzuklingen. In der überwiegenden Mehrzahl der Fälle bleiben sie apathogen. Nur selten erreichen Kälte-

[1] Mit Unterstützung durch die Deutsche Forschungsgemeinschaft (Schu 12[illegible] 10

agglutinintiter und hämolytische Aktivität bei diesen Pneumonien ein Ausmass, das zu den klinischen Symptomen von Kälteakrozyanosen, massiver Autohämolyse, Hämoglobinurie und schwerer Anämie führt [1, 3, 4, 4a, 5, 8, 9, 10, 13, 14, 15, 16, 20a, 21, 23, 24, 27, 28]. Trotz teilweise hochgradiger Anämie verläuft die hämolytische Erkrankung bei solchen Patienten meist gutartig und pflegt mit dem spontanen Rückgang des Kälteautoantikörpertiters innerhalb weniger Wochen auszuheilen. Von den bisher beschriebenen Fällen verliefen nur wenige tödlich [18, 20].

Soweit wir das Schrifttum übersehen, liegen von dieser Form der Kälteagglutininkrankheit noch keine ausführlicheren autoptisch-histologischen Untersuchungen vor. Über solche können wir hier anhand einer eigenen Beobachtung berichten.

Kasuistik

Sch., K., ♂, geb. 1923, Kriegsrentner. Der Patient befand sich wegen einer Psychopathie schon seit Jahren in einem schlechten Allgemein- und Ernährungszustand. Am 11. 3. 1966 erkrankte er zusätzlich an einem fieberhaften katarrhalischen Infekt mit starker Abgeschlagenheit, Husten, Auswurf und stechenden Schmerzen in der rechten Brustseite. Am 23. 3. bemerkte er eine braune Verfärbung des Urins. Gleichzeitig trat ein Sklerenikterus auf. Der Hausarzt vermutete eine Hepatitis und wies ihn am 14. Krankheitstag in das Krankenhaus Waldshut ein.

Klinische Befunde. Bei der Aufnahme war der Patient schläfrig und verwirrt und bot ein kachektisches Bild. Tiefhalonierte Augen. Exsikkotischer Eindruck. Blassbläuliche Verfärbung der Finger, Zehen und Nasenspitze. Deutlicher Skleren- und Hautikterus. Zunge trocken und weisslich belegt. Keine Lymphknotenschwellungen. Über der rechten hinteren Lungenbasis ca. 4 Querfinger hohe mässige Dämpfung, auskultatorisch in diesem Bereich mittelblasige, etwas klingende Rasselgeräusche. Links hinten unten einzelne Rasselge- [illegible] Hinfälligkeit des Patienten zu- [illegible] RR 80/60 mm Hg. Leber und [illegible]

Laboratoriumsbefunde. Hb 5,1 g%, Retikulozyten 35‰, Serumbilirubin 5,7 mg%. Urobilinogen im Urin kältepositiv, Eiweissreaktion positiv. Leukozyten 15900 mit mässiger Linksverschiebung. Blutsenkung 60/82 mm, später 110/140 mm. SGOT 21 mE/ml, SGPT 26 mE/ml.

Immunhämatologischer Befund. Das Patientenzitratblut zeigte bei Zimmertemperatur auf einem Milchglasobjektträger eine starke Autoagglutination, die bei Erwärmen auf ca. 40°C völlig verschwand, bei erneuter Abkühlung auf 20°C aber wieder auftrat. Das Patientenserum ergab mit O-I-Erwachsenenerythrozyten im Eiswasserbad nach 8 Stunden einen Kälteagglutinintiter von 2000, mit Nabelschnurerythrozyten einen Titer von 64. (Die gleichen Nabelschnurerythrozyten wurden von einem Anti-I-Serum mit dem O- [illegible] Nabelschnur- [illegible] °C, [illegible]

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Zusammenfassend handelte es sich klinisch um eine Mykoplasmainfektion, die offenbar durch eine bakterielle Pneumonie kompliziert war, bei dem schon vorher jahrelang kachektischen Patienten aber nur zu geringer Fieberreaktion führte. Außerdem wurden Kälteantikörper mit hohem Agglutinintiter und erheblicher autohämolytischer Aktivität gebildet, die vom 11. Krankheitstage an zu einer schweren hämolytischen Anämie mit Hämoglobinurie und Kälteakrozyanosen führten. Bei träger erythropoetischer Regeneration und anhaltender hochgradiger Anämie starb der Patient am 30. Krankheitstag.

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Der makroskopische Obduktionsbefund war uncharakteristisch. Sicher nachweisbar waren nur herdförmige pneumonische Infiltrationen, eine mäßige allgemeine Arteriosklerose, auch der Koronarien, und eine deutliche Dilatation des linken, leicht hypertrophierten Herzventrikels.

Demgegenüber erbrachte die mikroskopische Untersuchung der Organe eine Fülle von Veränderungen, die zum einen Teil dem spezifischen, immunologisch bedingten hämolytischen Prozeß und seinen unmittelbaren Folgen, zum anderen einem terminalen Kreislaufversagen und schließlich praeexistenten oder zusätzlichen Erkrankungen zuzuordnen sind. In die letzte Gruppe gehören die Hypertrophie der Muskelfasern der linken Herzkammer und die verbreitete Arteriolosklerose der inneren Organe. Die *Lungen* zeigen überall recht ausgedehnte, frische, konfluierende, stellenweise fibrinös-hämorrhagische Herdpneumonien. Interstitielle, an Viruserkrankungen gemahnende Entzündungsprozesse, wie sie bisher bei Mykoplasmapneumonien beschrieben worden sind [25], sind nicht zu erkennen. Man muss also annehmen, daß die ursprüngliche atypische Pneumonie spurlos abgeklungen war, und die jetzigen Entzündungen, erst vor kurzem entstanden, ein unspezifisches Ereignis darstellen, das freilich seiner Ausdehnung halber für den tödlichen Ausgang der Erkrankung von Wichtigkeit gewesen ist. An den roten Blutkörperchen sind hier wie an vielen anderen Organen bemerkte Veränderungen nachzuweisen, die eine immunologische Schädigung anzeigen könnten.

In *Leber* und *Milz* kann man in den geschwollenen retikulären Zellen sowohl eine beträchtliche Erythrozytenphagozytose wie eine erhebliche Siderose nachweisen. Diese Siderose ist in der Milz so ausgeprägt, daß ein eisengefärbter Schnitt schon dem bloßen Auge blau erscheint. Sie betrifft in erster Linie die Retikulumzellen der roten Pulpa (Abb. 1), nur vereinzelt sind auch einige retikuläre Elemente der aufgelockerten kleinen Lymphfollikel [illegible] Malpighischen Körperchen an der Eisenablagerung beteiligt. Dabei handelt es sich hier sowohl um eine diffuse Siderose wie um eine körnig-schollige Ablagerung eisenhaltigen Pigmentes. Die Form der Eisenablagerung und die Art der Erythrozytenphagozytose sind klarer und eindrucksvoller auch an der *Leber* zu sehen. Die mäßig vergrößerten Sternzellen sind durchweg erheblich geschwollen und so reich an Eisen, daß sie schon bei schwacher Vergrößerung deutlich hervortreten und das Balkenwerk [illegible] (Abb. 2). Sie weisen eine diffuse Blaufärbung auf, können aber auch gröbere und feinere Schollen

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räusche. Eine Röntgenaufnahme des Thorax war wegen der Hinfälligkeit des Patienten zunächst nicht möglich. Temperatur 37,6° C, Puls um 100/min, RR 80/60 mm Hg. Leber und Milz nicht vergrössert. Übriger klinischer Befund unauffällig.

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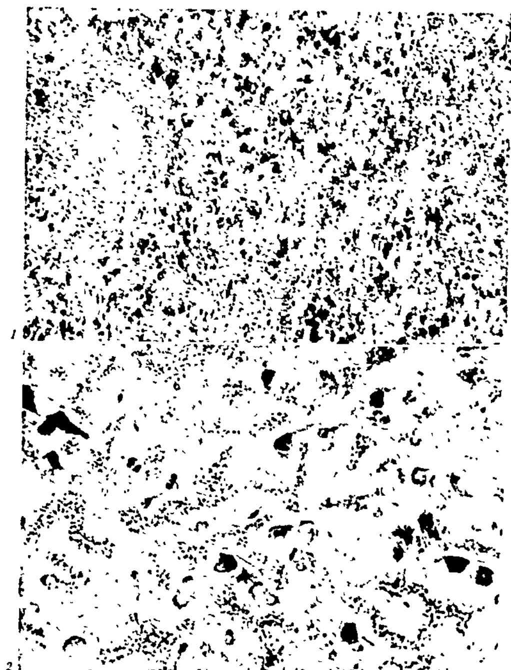


Abb. 1. Milz: Eisenspeicherung in den Retikulumzellen der roten Pulpa. Das links oben gelegene Malpighische Körperchen (mit Arteriolenhyalinose) ist weitgehend ausgespart (Turnbull-Reaktion, ca. × 190).

Abb. 2. Leber. Kräftige, diffuse bis grobschollige Eisenablagerungen in den stark geschwollenen und auch mässig vermehrten Sternzellen. Geringere, feinkörnige, peribiliäre Siderose in den Leberepithelien (Turnbull-Reaktion, ca. × 375)

enthalten (Abb. 3). Ausserdem enthalten die Sternzellen neben optisch leer erscheinenden Resorptionsvakuolen sehr oft Erythrozyten (Abb. 3a und b). Manchmal ist nur ein einziges rotes Blutkörperchen, manchmal eine grosse Zahl phagozytiert. Dass sie abgebaut werden, belegt ihre unterschiedliche Grösse und Färbbarkeit sowohl nach Lepehne wie nach Goldner. Nicht selten ist dann der nächstgelegene Teil des umgebenden Zytoplasmas durch eine besonders intensive Eisenreaktion ausgezeichnet, weshalb er als tiefblauer Ring in Erscheinung tritt (Abb. 3a). Einzelne Sternzellen haben sich aus dem Verband gelöst und liegen in der Zentralvene. Im Vergleich zu den Sternzellen trifft man in den Epithelien nur wenig histochemisch reagierendes Eisen, vor allem in den peripheren Läppchenarealen in Gestalt feiner, perilobulär gelegener Körnchen (Abb. 2). Als weitere Folge des Erythrozytenunterganges sieht man in den etwas weiten Gallenkapillaren sogenannte Gallethromben, daneben aber auch nicht gallig imprägnierte kräftig eosinrote Ausgüsse und in Lepehne-Präparaten sogar benzidinpositives Material, also Hämoglobin. Auch in den Leberzellen selbst und neben vereinzelten Galle- und Eiweissablagerungen gelegentlich charakteristisch gefärbte Hämoglobintropfen nachweisbar. Die inneren Läppchenschichten zeigen eine fein-

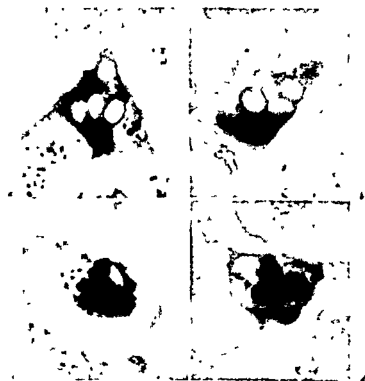


Abb. 3. Leber. Erythrozyten[illegible] und [illegible] in stark [illegible] Sternzellen. [illegible] Verteilung der Eisenreaktion in dem den Erythrozyten [illegible] Turnbull-Reaktion, × [illegible]

bis mitteltropfige Verfettung Sie ist als «degenerativ» zu werten und mit einer anämiebedingten Hypoxydose der Leberzellen zu erklären Demgegenüber sind die frischen Einzelzellnekrosen dem präterminalen Kreislaufversagen zuzuordnen

Auch manche Veränderungen der *Niere* sind durch den Kreislaufzusammenbruch bedingt Dazu gehören jene Phänomene, die zwar bei akuten tödlichen Hämolysen («Chromoproteinurie») [32] selten fehlen, ohne dafür pathognomonisch zu sein, da sie auch bei Kollapszuständen anderer Ätiologie vorkommen, weshalb sie generell als morphologisches Zeichen einer oligämischen Nephropathie angesprochen worden sind [2] Dabei handelt es sich um eine Erweiterung der Tubuluslichtungen, um eine feinwabige Beschaffenheit vieler stark geschwollener Hauptstückepithelien (Abb. 4, 5), um das Vorkommen von Einzelzell-

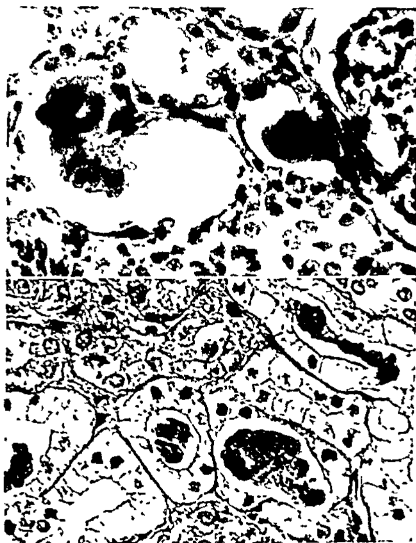


Abb 4 Niere Hämoglobinzylinder in Harnkanälchen *a* in erweiterten Mittelstücken, *b* in Hauptstücken mit stark geschwollenen, feinwabig hydropischen Epithelien *a* Gefrier Lepehne, *b* Paraffin Kernechtrot, × 480)

nekrosen im proximalen und distalen Tubulusabschnitt und schließt sich um das Auftreten von Oxalatkristallen. Daß diese Schädigungen beim Tode des Patienten noch nicht lange bestanden haben, daß es sich daher nur um recht akute Läsionen handelt, beweist das Fehlen jeglicher Regenerationserscheinungen. Demgegenüber läßt sich an den Glomerulusschlingen kein pathologischer Befund erheben. Manche Glomerula enthalten allerdings im Kapselraum etwas krümeliges Eiweiß gelegentlich auch (Lepehnereaktion: Hämoglobin). Um so reichlicher findet sich blaurot gefärbtes Eiweiß in den Tubuli, besonders in den distalen Anteilen. Ebenso finden sich granuläre Hämoglobinzylinder oder -schollen, die eine positive Lepehne-Reaktion geben und im ungefärbten Schnitt oder im HE-Präparat durch ihren charakteristischen braunroten Farbton auffallen. Auch die erwähnten nekrotischen und in die Tubuluslichtung abgestoßenen Zellformen können von Hämoglobin durchtränkt sein und dann die gleichen Farbreaktionen geben. Eine tubuläre Eiweißrückresorption ist jedoch nur selten und nur als umschriebene feinkörnige, allenfalls feintropfige Eiweißspeicherung nachweisbar. Bei der Eisenfärbung findet man – und zwar bei der Methode nach TIRMANN-SCHMELZER reichlicher als bei der einfachen Berliner-Blau-Reaktion – sowohl das blaugefärbte homogene Eiweiß wie das granuläre hämoglobinhaltige Material unter blaugrün (Abb. 6). An den nicht immer erhaltenen



Abb. 5. Niere. Eisenfärbung ... Tubu... Turnbull-Reaktion ...

bis mitteltropfige Verfettung Sie ist als «degenerativ» zu werten und mit einer anämiebedingten Hypoxydose der Leberzellen zu erklären Demgegenüber sind die frischen Einzelzellnekrosen dem präterminalen Kreislaufversagen zuzuordnen

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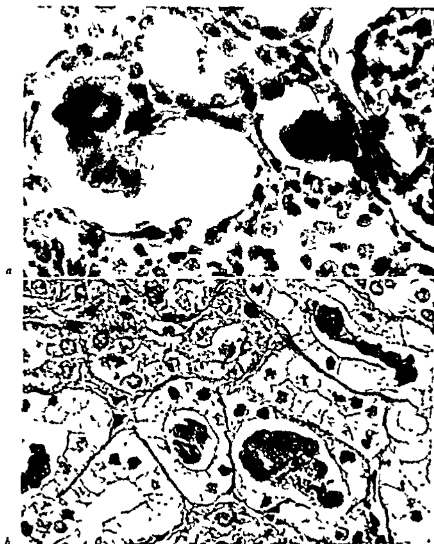


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nekrosen im proximalen und distalen Tubulusabschnitt und schließlich um das Auftreten von Oxalatkristallen. Daß diese Schädigungen beim Tode des Patienten noch nicht lange bestanden haben, daß es sich dabei mithin um recht akute Läsionen handelt, beweist das Fehlen jeglicher Regenerationserscheinungen. Demgegenüber läßt sich an den Glomerulumschlingen kein pathologischer Befund erheben. Manche Glomerula enthalten allerdings im Kapselraum etwas krümeliges Eiweiß, gelegentlich auch (Lepehnereaktion) Hämoglobin. Um so reichlicher findet sich Hämrot gefärbtes Eiweiß in den Tubuli, besonders in deren distalen Anteilen. Ebenso finden sich granuläre Hämoglobinzylinder oder -schollen, die eine positive Lepehne-Reaktion geben und im ungefärbten Schnitt oder im HE-Präparat durch ihren charakteristischen braunroten Farbton auffallen. Auch die erwähnten nekrotischen und in die Tubuluslichtung abgestoßenen Zellformen können von Hämoglobin durchtränkt sein und dann die gleichen Farbreaktionen geben. Eine tubuläre Eiweißrückresorption ist jedoch nur selten und nur als umschriebene, feinkörnige, allenfalls feintropfige Eiweißspeicherung nachweisbar. Bei der Eisenfärbung findet man – und zwar bei der Methode nach TURNBULL-SCHMELZER reichlicher als bei der einfachen Berliner-Blau-Reaktion – sowohl das Hämgefärbte homogene Eiweiß wie das granuläre hämoglobinhaltige Material mitunter blaugefärbt (Abb. 6). An den nicht immer erhaltenen



Abb. 6. Niere. Eine [illegible] Zylinder [illegible] von nekrotischen [illegible] Zellen [illegible] im distalen Tubulus (Turnbull-Reaktion [illegible] 900, [illegible])



Abb 6 Niere Eisenpositives Material in der Lichtung, am Bürstensaum und vereinzelt auch in den Epithelien der durchweg weiten Tubuli (Turnbull Reaktion, × 150)

Bürstensäumen der Hauptstücke sieht man oftmals feine, eisenhaltige Eiweisskrümel Gelegentlich sind auch in dem daruntergelegenen Zellabschnitt feine, resorptiv entstandene eisenhaltige Granula zu erkennen Intrakanalikuläre Erythrozyten fehlen

Diese Befunde beweisen, dass durch die Glomerula sowohl Hämoglobin wie gewöhnliches Eiweiss und histochemisch nachweisbares Eisen ausgeschieden worden sind Sie belegen damit eine intravasale Hämolyse und die Existenz einer Hämoglobinurie sowie einer Hämosiderinurie Allerdings kann die Menge des in den Tubuluslichtungen nachweisbaren Materiales, der präterminalen kollapsbedingten Minderung des Filtrationsdruckes wegen, nicht als Gradmesser für die Schwere und das Ausmass des hämolytischen Geschehens gelten

Diskussion

Der 13jährige Patient erkrankte an einer Pneumonie, als deren Erreger Mycoplasma pneumoniae durch die Komplementbindungsreaktion gesichert werden konnte Beim Tode war dieser Infekt schon abgeklungen Denn die jetzt noch nachweisbare konfluierende Bronchopneumonie hatte ein uncharakteristisches Aussehen Überdies zeigte sie ein akutes Bild und ist daher, zumal interstitielle entzünd-

liche Reaktionen fehlten, eher als eine sekundäre Komplikation aufzufassen, für die wahrscheinlich die klinisch nachgewiesenen »Begleitbakterien«, insbesondere die Influenzabakterien, verantwortlich zu machen sind. Im Verlaufe der initialen Mykoplasma-Infektion kam es zu einer begleitenden pathologischen Kälteantikörperbildung mit ausgeprägt pathogenem autoagglutinierenden und autohämolysierenden Effekt. Klinisch entwickelte sich eine schwere hämolytische Anämie mit Hämoglobinurie. Eine Benzidinreaktion war zwar vor dem Tod im Harn nicht durchgeführt worden, doch liess sich die Hämoglobinurie durch den histologischen Nierenbefund beweisen. Die Erkrankung endete am 30. Krankheitstag tödlich. Die Ursache für den ungewöhnlich deletären Verlauf ist durch das Zusammentreffen dreier Faktoren erklärt: einer schon seit Jahren bestehenden allgemeinen Kachexie im Rahmen einer Psychopathie, der primären und sekundären Pneumonie und der schweren autoimmunhämolytischen Anämie. Wie das histologische Bild der Niere zeigt, ist der Tod auf ein mit Blutdruckabfall einhergehendes Kreislaufversagen zurückzuführen.

Immunhämatologisch liess sich bei einem Kälteagglutinintiter von 2000 ein Hämolysintiter von 8 (bei 20 C Reaktionstemperatur) nachweisen. Eine solche Konstellation mit ausgeprägter hämolytischer Aktivität bei *relativ* niedrigem Kälteagglutinintiter scheint – abweichend von chronischen Kälteagglutininkrankheiten – für postpneumonische hämolytische Anämien durch Kälteautoantikörper charakteristisch zu sein. Da dem Kälteagglutinintiter von 2000 mit I-positiven Testerythrozyten von Erwachsenen ein *relativ hoher Kälte*agglutinintiter von 64 mit nahezu I-negativen Nabelschnurerythrozyten entsprach, ist anzunehmen, dass die vermehrten Kälteagglutinine des Patienten entweder aus einem inhomogenen Gemisch von anti-I und anti-i spezifischen Antikörpern bestanden oder innerhalb des I-i-Systems keine scharfe Spezifität aufwiesen. (Weitere Spezifitätsuntersuchungen waren wegen der verfügbaren geringen Serummengen nicht möglich.) An der pathogenen Bedeutung der Kälteautoantikörper für die schwere hämolytische Anämie kann aufgrund der klinischen und immunhämatologischen Befunde kein Zweifel bestehen. Ihr autozytotroper Effekt wurde an den Patientenerythrozyten durch den positiven direkten Antiglobulintest vom »Komplementtyp« nachgewiesen. Diese Variante des Coombs-Tests entspricht einer hämolytisch unterschwelligen Schädigung der Patientenerythrozyten, die dann, wie der

histologische Befund zeigt, von den Retikulumzellen in Leber und Milz *phagozytiert* werden Dass diese Erythrozytenphagozytose zum Zeitpunkt des Todes noch im Gange war, beweist das intrazelluläre Vorkommen scheinbar unversehrter, also frisch aufgenommener roter Blutkorperchen (Abb 3) Fur die klinisch nachgewiesene langere Dauer des Prozesses ist der Reichtum hamoglobinogenen Eisens vor allem in Form des scholligen Hamosiderins, ein eindrucksvolles histologisches Zeichen

Die gleichzeitige, bei der Turnbull Reaktion besonders deutliche Eisenablagerung in Leberepithelien spricht dafür, dass auch der Serumeisenspiegel betrachtlich erhoht war Als Voraussetzung dafür ist eine intravasale Hamolyse anzusprechen die sich morphologisch am besten aus den scholligkornigen Hamoglobinzylindern der Niere (Abb 4) erschliessen lasst Ihre grosse Zahl ist wahrscheinlich mit der praterminalen Niereninsuffizienz zu erklaren Fur das eisenfärbbare Material in der Lichtung und am Burstensaum der Tubuli gilt das gleiche Sonst hatte man wohl auch, besonders nach den Erfahrungen bei chronischer Kalteagglutininkrankheit [26] eine starkere Eisenspeicherung erwarten mussen Denn die für eine auffallend geringe oder gar fehlende tubulare Eisenresorption bei akuten hamolytischen Bluttransfusionszwischenfallen gultige Erklarung [32] – eine schadigungsbedingte Resorptionsinsuffizienz – kann in unserem Falle kaum ins Feld gefuhrt werden Sprechen doch die klinischen und die morphologischen Beobachtungen dafür dass die zweifellos vorhandene tubulare Schadigung beim Tode des Patienten erst kurze Zeit bestanden hat

Die morphologischen Befunde, soweit sie der immunologisch bedingten Erythrozytenschadigung zuzuordnen sind entsprechen grundsatzlich jenen, die wir vor Jahren bei der chronischen Kalteagglutininkrankheit mitgeteilt haben [26] Doch sind sie im vorliegenden Fall wegen der geringeren Schwere und der geringeren Dauer, weniger ausgepragt und weniger fortgeschritten Aus dem gleichen Grunde treten hier auch solche Veranderungen die als direkte Folge einer schweren langeren Anamie bekannt sind in den Hintergrund Die sekundaren «degenerativen» Nierenveranderungen die Zellschwellungen und Zellnekrosen etwa, sind gleich denen des von uns fruher beschriebenen Falles chronischer Kalteagglutininkrankheit Doch nur in jenem sind sie eine zwar *mittelbare aber alleinige Folge der schweren hämolytischen* Anamie und damit der reine Ausdruck einer sich todlich

steigernden «Chromoproteinurie» im Sinne ZOLLINGERS [32]. Bei unserem Patienten aber sind diese Befunde nur darauf zurückzuführen, dass der Erkrankte durch schwere Kachexie vorgeschädigt und durch konfluierende Bronchopneumonien zusätzlich belastet war. Der Tod des Patienten ist also nicht ausschliesslich der akuten autoimmunhämolytischen Anämie, sondern ihrem Zusammenwirken mit anderen ungünstigen Einflüssen zur Last zu legen.

Zusammenfassung

Die akute, durch Kälteautoantikörper bedingte hämolytische Anämie nach Mykoplasma-Infektion verläuft nur äusserst selten tödlich. Über einen solchen Fall wird hier erstmals mit ausführlichen autoptisch-histologischen Befunden berichtet. Bei einem Kälteagglutinintiter von 2000 und ausgeprägter hämolytischer Aktivität der Kälteautoantikörper kam es zu einer schweren hämolytischen Anämie mit Hämoglobinurie. Autoptisch fanden sich als Folge des autoimmunhämolytischen Prozesses die Zeichen der Hämoglobinämie und Hämoglobinurie sowie einer unterschwelligen Blutkörperchenschädigung mit Phagozytose scheinbar noch intakter Erythrozyten durch retikuläre Zellen der Milz und Leber. Im Prinzip gleichen die Veränderungen denen bei chronischer Kälteagglutininkrankheit nach tödlich verlaufenen Krisen.

Summary

Fatal course of acute haemolytic anaemia caused by cold agglutinins after mycoplasma infection is a very rare condition. Such a case is reported here, including for the first time extensive histological findings at autopsy. The cold agglutinins with a titre of 2000 and a strong haemolytic activity led to severe haemolytic anaemia with haemoglobinuria. At autopsy there was evidence of haemoglobinaemia and haemoglobinuria and of phagocytosis of altered but not yet haemolysed erythrocytes by reticular cells of the spleen and liver. These findings are comparable to those seen after a fatal crisis of chronic cold agglutinin disease.

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Haemoglobin D-Thalassaemia

A Case Report

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The existence of haemoglobin D has been reported in the Indian population of Punjab and Gujrat [5]. The incidence of haemoglobin D among American Negroes was found to be 0.4% in North Carolina, 0.085% in Baltimore and 0.20 to 1.9% in Latin America. Isolated cases of haemoglobin D have been encountered in Turks [1], Britain, Algeria, the Belgian Congo, the Philippines, and American Indians. The majority of heterozygote and homozygote haemoglobin D carriers enjoy reasonable health. However, a few cases of haemoglobin D-thalassaemia have been reported [2, 7, 8]. Therefore, looking towards the rarity of the condition it is thought germane to report this case.

Methods

The foetal haemoglobin was estimated by the method of alkali denaturation for 1 min. The solubility test was done as described by Itano. Sickling tests were done by the method of Daland and Castle. Electrophoresis of haemoglobin was carried out on filter paper at pH 8.6 with barbital buffer on a current 150 V for 16 h. Other haematological investigations were done in the usual manner.

Case Report

Abha, 1½ years, a female child was referred from paediatric ward to the Department of Pathology, SMS Medical College, Jaipur on 2.11.68 for the evaluation of anaemia and weakness. On physical examination Abha was found to be of thin build. She had some degree of mongoloid features with pallor of skin and mucous membranes. Icterus was not seen. The abdomen was distended, both liver and spleen were enlarged 4 cm and 9 cm respectively below the costal margins. The roentgenogram of skull revealed some degree of thickening of the diploe. The haematological findings of the patient and her family are

shown in table I. The electrophoretic pattern of haemoglobin is shown in figure 1. These findings led us to think about the thalassaemia.

The past history of the parents revealed the death of two siblings 5 years and 3 years ago the cause of which could not be judged.

Discussion

The parents of the patient Abha belong to a Sindhi family having migrated from Punjab. Punjab area is supposed to be reservoir for haemoglobin D_{Punjab} or $D_{Los\ Angeles}$ [3, 4]. Homozygotes or heterozygotes of haemoglobin D alone are usually asymptomatic. The association of haemoglobin D with other abnormal haemoglobins results in pathological conditions. One sibling of the family investigated by us seems to be a case of Hb D thalassaemia or Hb D-disease, where the father has transmitted the Hb D gene and the mother the thalassaemia gene forming a double heterozygous condition. Another sibling inherited only the thalassaemia gene from the mother. Two siblings died of severe anaemia at the ages of 3 and 2 years. Detailed

Table I. Haematological data of the patients and the members of his family

	Patient	Father	Mother	Brother of propositus
Age years	1.5	35	27	10
RBC $10^6/mm^3$	3.0	5.0	4.5	5.0
Hb g%	7.0	13.5	11.8	[illegible]
PCV %	30.0	40.0	33.0	40.0
MCV μm^3	[illegible]	80.0	81.0	85.0
MCH pg	23.3	27.0	26.2	27.5
MCHC %	31.0	33.0	29.4	31.2
Reticulocytes %	8.0	1.5	3.8	1.0
Target cells	many	non	many	few
RBC morphology	hypochromia anisocytosis, poikilocytosis	normochromic	hypochromic	hypochromic
Nucleated red cells in peripheral blood	many		few	few
Osmotic fragility % NaCl	0.55-0.15	0.45-0.25	0.40-0.20	0.40-0.20
Sickling test	neg	neg	neg	neg
Foetal Hb %	3.0	0.2	1.80	1.1
Alkali resistant Hb %	21.0	1.0	13.5	[illegible]
Electrophoretic pattern of Hb	DD	DD	AA	AA
Blood group	O Rh+	O Rh+	O Rh+	O Rh+

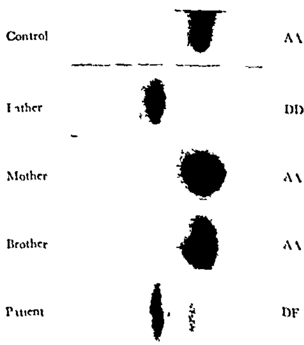


Fig 1 Electrophoretic pattern of haemoglobins in a Sindhi family

investigations were not carried out in them Probably those children might have had Hb D-thalassaemia or Hb D-disease.

Occasional patients have been reported who are heterozygous for both β-thalassaemia and one of the Hb D variants Since the Hb D amino acid substitutions may involve either the α- or the β-chain, two types of syndromes associated with Hb D-β-thalassaemia can be expected and indeed both have been found Individuals heterozygous for Hb D-β-thalassaemia probably represents the association between β-thalassaemia and an α-chain substituted haemoglobin [6, 9] The pattern of inheritance of these two abnormalities again confirms the lack of genetic linkage between the loci determining α- and β-chain abnormalities

Acknowledgement The authors are thankful to Prof J B CHATTERJEA, School of Tropical Medicine, Calcutta *for guiding as regards the confirmation of Hb D*

Summary

A Sindhi family with Hb D thalassaemia is described One child presents a combination of Hb D and β-thalassaemia while another child reveals only β-thalassaemia Father and mother has Hb D and β-thalassaemia minor respectively

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Cellular and Humoral Mechanisms in Anaphylaxis and Allergy. Proceeding of the 3rd International Symposium of the Canadian Society for Immunology, Toronto, Oct 3 5, 1968 MOVAT and HENRY (ed) Karger, Basel/München/New York 1969 X+150 p., 51 fig , 30 tab , sFr /DM 71 –/US $ 17 05/142 s

Cellular immunity and transplantation are the most fashionable topics in immunology today Allergy, mediated by antibodies, however, might have much more far reaching importance, at least in clinical medicine Only during recent years clearer concepts about some of the mechanisms involved have emerged Details are accumulating fast and it is difficult to keep up with them and to integrate them into a more general concept This volume is the result of a symposium at Toronto in 1968 Though another symposium dealing with a similar subject has been edited by K F AUSTEN and E. L. BECKER (*Biochemistry of the Acute Allergic Reaction* 1968), with some of the authors participating in both of them, the present volume is very useful for its additional data and its concise presentation It has 5 sections, all but one opened by a short review of the topic to be treated, which is helpful for following many of the results presented in the subsequent papers The first section deals with 'antibody in anaphylaxis and allergy', and offers many details about reaginic, homocytotropic and heterocytotropic antibodies and their characteristics The cells activated by these antibodies are further considered in the next section on 'cellular events in anaphylaxis,' together with some of their products This is followed by 2 papers on slow reacting substance The humoral mediators generated in plasma, the kinins and anaphylatoxin are described in the last 2 sections

In a way, more questions are asked than answered But new specific inhibitors of some of the mediators and a closer look at the experiences made in the field of coagulation might, as some of the authors suggest, bring an even faster development in the future This book will not give definite answers but puts together what is known and what is not yet known and should stimulate immunologists particularly concerned with lymphocytes clinical allergists and those dealing with coagulation, and it provides a reference source for those already working in the field

T L. VISCHER, *Basel*

A NOVOTNY **Basic Exercises in Immunochemistry. A Laboratory Manual.** Springer, Berlin/Heidelberg/New York 1969 VIII+197 p , 50 fig , DM 38 –/US $ 9 50

Since the publication of 'Methods in Immunology by D H CAMPBELL *et al*, no practical methodological textbook aimed at helping the beginner or the design of teaching courses has appeared This laboratory manual by A NOVOTNY is a very useful addition for it introduces students and novices with some biochemical background to the most frequently occurring problems in immunochemistry It has 3 parts, one on isolation methods one on structural studies and the last on immunological and other biological assays, each containing about 20 exercises and giving examples of most of the more important areas. The introduction to each exercise contains a brief and elementary explanation of the reaction, 'materials and equipment' lists the chemicals, biologicals and the special equipment necessary to carry out the exercise 'Procedure' gives simple and short instructions with sufficient details that the student without particular experience can carry it out with success Then, help is provided to evaluate the results and most important the applications and limitations of each method is shortly discussed and reference made to related procedures in another exercise of the book A few pertinent references are furnished with each exercise

This laboratory manual is concise, well presented and deserves to be in every teaching laboratory In addition it is useful for consultation for even more experienced workers who must tackle problems in fields in which they have little personal experience

T L. VISCHER, *Basel*

Acta haemat. 44: 129–141 (1970)

The Mixed Lymphocyte Tumor Cell Culture and the Fluorescein Diacetate Cytotoxicity Test

Two Useful Methods for the Demonstration of Cell Mediated Tumor Specific Immunity in Man[1]

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The search for human cancer specific antigens and immune mechanisms which might regulate the host tumor relationship has been vigorously reactivated during the past decade. This is a consequence of the demonstration in animal systems of tumor specific immunity, which covers not only basic conceptions such as specific cell antigens and antibody mediated and cell mediated host responses, but includes also such phenomena as immunosuppression, immunoselection, antigenic modulation, allogeneic inhibition and immunologic tolerance or paralysis. The abundant literature has been comprehensively reviewed [5, 12, 13, 25, 26]. Despite mounting evidence of specific antigens in human neoplasms few facts are irrefutably established because of the inherent limitations of clinical investigation and the paucity of reliable in vitro methods. Positive evidence was mainly obtained with direct or indirect immunofluorescence, complement fixation, immune adherence, immunodiffusion, cytotoxicity with immune sera, immunological tolerance and absorption techniques. These techniques were designed to demonstrate either circulating tumor antibody or cell fixed antigen. (Summarized by various authors in Cancer Research, vol 23, July 1963.)

The effector cells in delayed hypersensitivity, in host versus graft reactions against normal tissues and against tumors transplanted in

[1] This investigation was supported by Public Health Service Research Grant No. CA [illegible] from the National Cancer Institute.

[2] Chief of Medicine A and Director Cancer Clinical Research Center, RPMI.

immune animals are lymphocytes [7, 8, 19]. In man cell mediated immunity has been studied exclusively in cytotoxicity tests. The principle of such tests is the deleterious effect of immune lymphocytes on target tumor cells, as first described by ROSENAU and MOON [22]. The colony inhibition test, in which the plating efficiency of neoplastic is measured after their incubation with lymphocytes from immune donors, has proved to be particularly valuable [10]. Other arguments favoring the role of lymphocytes as the effector cell in transplantation immunity derive from the mixed lymphocyte culture (MLC) [4]. Blastoid transformation, cell division, metabolic activation and accelerated synthesis of nucleic acids are some measurable parameters occurring in the presence of allogeneic leucocytes. It is assumed that this reaction represents the recognition step of the homograft rejection response to antigens controlled by the major histocompatibility locus in man (HL-A) [1, 2].

Since a tumor with specific antigenicity can be regarded as a transplant in a given host, the aim of the present study was to prove whether those principles established in histocompatibility testing for normal antigenic disparity could also provide a useful model to detect foreign, i.e. tumor specific antigenic structures. Two systems were used which have not previously been reported for application to tumor immunology: (1) The mixed lymphocyte tumor cell culture (MLTC) which indicates by blastoid transformation of lymphocytes both, the presence of foreign antigens and the existence of immunocompetent lymphocytes to recognize it. (2) The fluoresceindiacetate (FDA) test previously described for tissue typing [6] in which fluorescein is released from target cells exposed to the cytotoxic activity of immune lymphocytes.

Our recent observation of congruent contemporaneous concordance of malignant melanoma in identical brothers of a set of triplets [27] provided us with one of the tumors which ranks high in the list of *human neoplasms for which evidence of immunity is accumulating* [14] and a unique experimental tool mimicking transplantation experiments in inbread strains. In addition, immediate family members of the triplets, another patient with widespread metastatic melanoma and an unrelated control were included in this study.

Materials and Methods

D. RPMI No. 124-168. An infiltrative growing malignant melanoma of the left anterior chestwall was removed at age 53. Four and one half year later, after several episodes of

tumor recurrence and reoperation, he died with widespread metastasis, predominantly pulmonary. *Tumors were obtained 3 weeks before death.*

T. RPMI No. 124 183. The monozygous triplet of D was found to have malignant melanoma of the same localisation at the same time of life as D. After operation by the same surgeon he was found to be clinically tumor-free on elective admission 4½ year later.

I. The fraternal triplet of D and T never had melanoma and was healthy at the time of our studies.

H. 64-year-old healthy elder brother of the triplets.

L. 15-year-old healthy son of T.

MS. RPMI No. 118-546, a 43-year-old white patient with malignant melanoma of the skin diffusely metastasizing predominantly to both her eyes, the lungs and the bone marrow. The qualitative differential count of marrow showed 96.8% tumor cells, 2.4% myeloid precursors and 0.8% lymphocytes.

N. 33-year-old white, healthy donor of control lymphocytes, unrelated to any of the other subjects.

Experiments were done under sterile precautions with siliconized equipment. The culture medium throughout was RPMI 1640 [16] supplemented with 20% fresh autologous plasma. Antibiotics were omitted.

Preparation of lymphocytes. 60 to 250 ml of peripheral venous blood was drawn and sedimented with 30 U/ml of freshly prepared preservative-free heparin at 37 °C for variable times according to the color of the supernatant plasma indicating minimal red cell contamination. The plasma was withdrawn and centrifuged for 10 min at 200 *g* to obtain leucocytes. The supernatant plasma was separated, rendered cell free by centrifugation for 10 min at 1 000 *g* and used in individual batches for the preparation of culture medium.

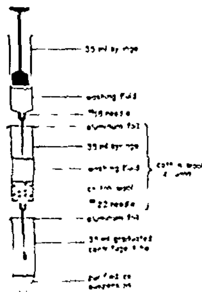


Fig. 1. Arrangement of materials for the purification of lymphocytes.

The leucocyte pellet was resuspended in 10 ml of culture medium and immediately incubated at 37°C in a 15 ml column for the absorption of granulocytes The column consisted of a 35 ml autoclavable plastic syringe A material like cotton wool was available (Johnson and Johnson Co as cleaning wool for xerox copy machines), in parallel, nearly of uniform diameter fibers in sheets of 5–8 mm thickness Strips were cut and rolled into loosely packed cylinders weighing approximately 3 g conveniently filling the lumen of the syringe up to 15 ml The column holds 13 ml of fluid After 30–45 min of incubation at 37°C the column was eluted with 30 ml warm culture medium at a flow rate of 1 ml/min regulated by the lumen of the outflow needle (gauge 22) (fig 1) This column removed generally more than 95% of the *polymorphonuclear cells originally present* The purification procedure was used to increase the accuracy of the method Exact quantitation of lymphocytes was possible since no clumps were formed with granulocytes Intimate contact of lymphocytes and tumor cells could occur without barriers of non lymphoid cells The possibility of toxic substances or enzymes being released by disintegrating cells was diminished and a source of thymidinphosphorylase activity, which was found especially high in polymorphonuclear leucocytes [15], was reduced After the eluate had been washed once and resuspended in culture medium, a small quantity was removed for counts in a Neubauer chamber and for Wright Giemsa stained smears for differentation

Preparation of tumor cells Tumor cells of patient D were obtained by needle aspiration of approximately 3 ml of fluid from a soft cystic subcutaneous tumor mass The cells were concentrated by centrifugation for 10 min at 200 *g* They were exposed twice for 20 sec to an osmotic shock with distilled water and resuspended in culture medium They were counted after vigorous pipetting with a Pasteur pipette, smeared and stained with Wright Giemsa stain Differential counts of the tumor cell suspension revealed 96% tumor cells and 4% leucocytes Viability tests were performed with FDA revealed 76+5% and with Trypan blue 82+3% viable cells

Tumor cells of patient MS were obtained by bone marrow aspiration from the iliac crest Approximately 4 ml of marrow were aspirated into a syringe containing 1 ml of Heparin 1,000 U/ml The aspirate was transferred to 4 test tubes containing 3 ml culture medium each, pipetted vigorously with Pasteur pipettes, and treated thereafter by 2 repeated hypotonic shock procedures as decribed above Differential counts revealed 96% tumor cells, 4% immature marrow elements, and red cell ghosts

The mixed lymphocyte tumor cell culture (MLTC) Within each experiment the number of the 'responding' lymphocytes was kept constant at a concentration of 2×10^6 cells per culture tube in the experiments with D tumor cells, and 1×10^6 lymphocytes in the experiments with S tumor cells Replicate lymphocyte tubes were prepared to which tumor cells as stimulating cells were added from serial dilutions, in equal volume but decreasing concentrations, giving final lymphocyte tumor cell ratios of 1 4, 1 2, 1 1, 2 1, 5 1, 10 1, 25 1, 50 1, 100 1 The suspensions were well mixed by pipetting in the 2.5 ml of culture medium and were cultured in 15/125 mm Pyrex test tubes where they sedimented and formed well defined pellets The tubes were tightly sealed with silicone rubber stoppers and kept at 37°C for 144 h static in the upright position A slight change in color toward yellow after 72 h indicated acid production in some tubes, but readjustment of the pH was not made For the last 5 h of the culture period ^{3}H-Thymidine 2 µC (spec Act 1 9/ Ci/mM) was added To terminate the experiment unlabelled thymidine was added in 1,000 × excess in order to dilute the uptake of the isotopic compound DNA was precipitated with 5% trichloroacetic acid according to the method of BACH [3] The acid insoluble material was thereafter dissolved in 0.2 ml phenethylamine and 15 ml liquid scintillation solution[1] and counted in a TriCarb spectrometer with correction for quenching The results are expressed in median counts per minute (cpm) of experiments done in triplicate

[1] PPO 7 g Dimethyl POPOP 0 3 g Naphtalene 100 g, Dioxane q.s ad 1,000 ml

The mixed lymphocyte culture (MLC) was carried out in these and similar studies as parallel and control experiments to prove the responsiveness of lymphocytes to allogeneic cells, and to give a guide line of the extent of the lymphocyte response in the presence of allogeneic cells with a known mitogenic capacity. The 'one way' modification of the MLC [3] was chosen because it allows the selective blastoid transformation of only one cell population of lymphocytes which can be measured as incorporation of ³H TdR into DNA. The 'stimulating' population was treated for 20 min with Mitomycin C, 0.05 mg/ml, and then was washed twice with culture medium. As responding cells 1×10^6 untreated lymphocytes were used. Mitomycin C treated cells were added to give a final responding: stimulating cell ratio of 2:1, 1:1 and 1:2 which in our experience reflects the optimal response range of the MLC.

Controls to test for spontaneous transformation the following cell preparations were cultured alone without further mitogens added (4×10^6 cells/tube): lymphocytes, Mitomycin C treated lymphocytes, tumor cells. Parallel controls were carried out with PHA (Phytohemagglutinin P, Difco, (2×10^6 cells/tube, 72 h culture period)): lymphocytes, Mitomycin C treated lymphocytes, tumor cells. In addition mixtures of Mitomycin C treated together with untreated autologous lymphocytes and mixtures of allogeneic lymphocytes all treated with Mitomycin C were included. To test the possibility that tumor cells in these culture conditions could be stimulated to synthesize DNA, they were exposed to allogeneic leucocytes which were treated with Mitomycin C (lymphocyte: tumor ratio 1:4). Culture conditions, media, labelling and the processing of the cell precipitate thereafter were identical to those of the MLC and the MLTC. Bacteriological controls of all cell suspensions before distributions to the final culture were set up and observed during the time of the cultures. Bacteriological controls at random were done from individual test tubes after the culture period. All remained sterile.

The fluorescein diacetate cytotoxicity test (FDA test). FDA labelled tumor cells were used as target cells to which autologous, syngeneic or allogeneic lymphocytes were added in vitro. The test demonstrates that malignant melanoma cells were able to hydrolyze the fluoresceindiester, and to accumulate free fluorescein intracellularly. Since fluorescein could not exit from the viable cell any toxic action of immune lymphocytes on tumor cells was indicated by disappearance of the fluorescence. The biochemical background of the reaction has been discussed elsewhere [23].

Labelling: 5 mg FDA were dissolved in 1 ml of analytical acetone to give a stock solution which was kept at 4 °C. The working solution was prepared daily dissolving 0.005 ml of the stock solution in 10 ml of phosphate buffered physiological saline. Cells to be labelled were resuspended in 1 ml working solution and incubated for 15 min at room temperature. After this time a greenish appearance of the suspension indicates the presence of fluorescent substrate outside the cells. The cell suspension thereafter must be repeatedly washed until the fluid remains clear. Only viable cells were labelled and appeared as bright fluorescent bodies on the black background of the dark field condenser under the UV-microscope. By changing the light source from UV to bright light, the total cell number was counted and the percentage of viable cells expressed as the ratio % labelled cells to total cells. Care must be taken to keep the labelled cells in the dark and protected from UV irradiation when not under the microscope.

In the present experiments responding lymphocytes were either prepared fresh as described above or they had been preincubated with PHA for 3 days or preincubated with PHA and 1% tumor cells for 7 days. Before mixing such preincubated lymphocytes with tumor cells they were counted and tested for viability by means of FDA uptake and adjusted to 2×10^6 viable cells/ml.

Tumor cells as target cells were labelled thereafter with FDA as described, mixed with unlabelled lymphocytes giving a lymphocyte: tumor cell ratio of 10:1, immediately counted and kept in test tubes at 37 °C in the dark. Small samples were withdrawn after

2, 4, 8, 12 and 24 h Two entirely independant replicate samples were withdrawn and read at each time point. The results are median values expressed in % viability

Leucocyte antigen typing HL-A typing of leucocytes was done in another laboratory[4]. This analysis was done as a further attempt to find a correlation between the degree of transformation in the MLC and MLTC by specifying antigens known to be involved in the lymphocyte transformation process [11]

Results

Positive stimulation is defined as the accumulation of over 2,000 cpm in at least 2 cell ratios tested. All other conditions are considered negative. The results with the MLTC with D tumor cells are given in figure 2 and with SM tumor cells in figure 3. No significantly elevated ^{3}H-TdR incorporation into DNA was observed in either patient in the presence of his own tumor cells. The lymphocytes were able to respond, however, to the stimuli of allogeneic cells. D lymphocytes responded to Nm (m=Mitomycin treated) leucocytes as well as his identical brother T did (fig. 4). SM lymphocytes responded to Nm leucocytes at all ratios tested (fig. 5). Positive stimulation was obtained with tumor cells in all other combinations. Of special interest are the different cell mixtures of the triplets. Whereas no mutual stimulation

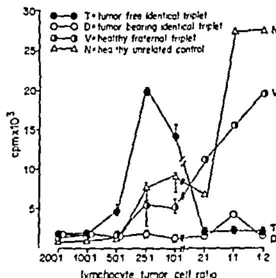


Fig 2 2 × 10^6 lymphocytes/tube stimulated with different concentrations of D tumor cells giving the ratios as indicated Positive stimulation of T at 3 different ratios

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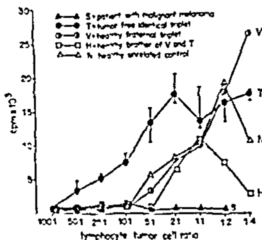


Fig. 3. 1.0×10^6 lymphocytes/tube stimulated with different concentrations of tumor cells giving the ratios as indicated. Stimulation with S tumor cells. Positive stimulation in allogeneic mixtures only.

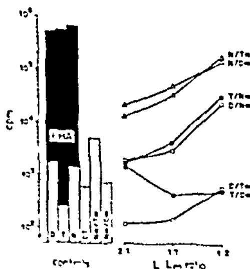


Fig. 4. The MLC: comparable mutual response of S to D and T, of D and T to S. Incorporation of DNA synthesis by M... C. Positive PHA cultures.

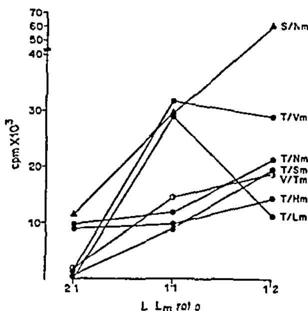


Fig 5 The MLC Different responses pattern of the leucocytes to allogeneic leucocytes

was found in the isogeneic leucocyte combinations (fig 4) the tumor free triplet E responded clearly to D tumor cells (fig 2) with positive stimulation at the Ly tu ratio 50 1, 25 1 and 10 1 which thereafter returned to background T also behaved differently from the other combinations with S tumor cells (fig 3) inasmuch he responded at much lower concentrations of tumor cells, whereas his response to Sm leucocytes (fig 5) is comparatively weaker and found only at higher concentrations of leucocytes as stimulating cells Comparing the leucocyte antigens (fig 7) of S, T, V, and H it is obvious that H differs from S in 4 groups, T and N in the same 3 groups each, and V in 2 groups only D and T type identically. Comparing the lymphocyte response of T in figure 5 in the different test combinations with the HL-A typing report (fig 7) no clear correlation is found between the order of magnitude of thymidine incorporation and the sequence of antigenic disparity

The mixture of T/Sm, where marked antigenic difference exists, gives lower counts than does the T/Nm combination who are antigenetically similar This is especially prominent when the Ly Lym ratios 2 1 are considered with T responding significantly to N, but not to

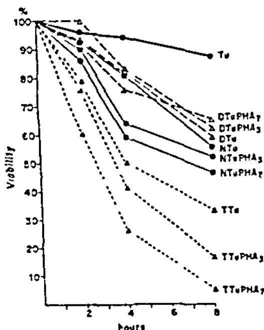


Fig 6. The FDA test. Tu: tumor cells alone. DTu, TTu, NTu: fresh lymphocytes with tumor cells. DTuPHA 3, TTuPHA 3, NTuPHA 3: Lymphocytes preincubated with PHA P for 3 days. DTuPHA 7, TTuPHA 7, NTuPHA 7: Lymphocytes preincubated with PHA P and 1% tumor cells for 7 days.

S. In all instances a dose relationship can be noted in the pattern of the individual curves. Increasing counts are obtained with an increasing amount of antigen (cells) added until a peak response is reached which is followed in some mixtures by a decline.

The T response to tumor cells (fig 2 and 3) shows that a peak is obtained much earlier than in the other cellular mixtures. A decline is found thereafter in figure 2, but nearly a plateau exists in figure 5. The FDA test was carried out over a period of 21 h. Repeated samples were still drawn from the lymphocyte-tumor stock tube and counted. The label at 12 h and thereafter had become very weak in all tumor cells including the control which made exact quantitation impossible. The results are therefore given only up to 8 h (fig 6). Over [illegible]% of the control cells remained stained at 8 h. In all other mixtures there was a steady decrease in viability most markedly expressed in the T tumor cell

Initials	Lymphocyte Antigens International HL-A Nomenclature												
	HLA 1	HLA 2	HLA 3	B 4	HLA 5	HLA 7	HLA 8	B 9	B 11	B 6	B 10	B 12	ABO
S	+	−	+	−	−	−	+	−	−	±	−	−	A
D	+	+	−	−	−	+	−	−	−	−	−	−	O
T	+	+	(−)	−	−	+	(−)	−	−	(−)	−	−	O
V	+	+	(−)	−	+	Δ	(−)	−	−	±	−	−	A
H	(−)	+	(−)	+	+	−	(−)	−	+	(−)	−	−	A
N	+	+	(−)	−	−	Δ	(−)	−	−	(−)	−	±	O
L	+	−	−	−	+	−	−	−	−	±	−	−	A

Fig 7 Leucocyte antigen typing report. Identity of D and T ○ indicates antigens S lacking Δ indicates antigens D/T lacking

mixtures Statistical analysis was done to compare the mixtures tu, D/Tu PHA3, N/Tu PHA3 and T/Tu PHA3 with each other Sampling and counting errors of 32 observations were taken into account Analysis of variance has shown that the cell mortality curves are not likely to be straight lines This implies that the mortality rate in a given preparation was not constant over the whole duration of the experiment. To compare the effects of the different varieties of lymphocytes on the mortality of tumor cells we used the slope of the best fitting straight line, so that the sum of the squared deviations of the data from the fitted line is minimal The hypothesis of equality of all 4 averaged rates (Tu-, D, N, T) is rejected by the F-test ($p > 0.001$) Individual t tests for each of the possible 6 pairs give the following results every pair is significantly different at the level of 1% except the pair N/tu PHA3 and D/tu PHA3 for which no difference could be shown No statistical analysis of the 3 individual mixtures (0, PHA3, PHA7) was done because it seemed impossible to take all errors into account Such an error might derive from the cell mixtures being adjusted to 2×10^6 viable cells This implies that in the mixture T/tu, PHA7 many more dead cells and their by-products were present which might themselves have a cytotoxic effect. It should be pointed out, nevertheless, that in 2 mixtures, the incompatible combination N/tu and the isogeneic mixture T/tu, the same order of cytotoxicity is found

Among the control cultures PHA stimulated leucocytes were positive. No stimulation of tumor cells occurred with PHA. All other controls were negative.

Discussion

A scientific group of the World Health Organization proposed in 1966 to study the technique of lymphocyte transformation *in vitro* for the detection of tumor specific antigens in identical twins, the only situation in man comparable to inbred animal strains [20]. We attempted these experiments to demonstrate cell bound tumor specific immunity in man in a variety of well selected clinical situations with two different methods not previously described for this purpose, the MLTC and the FDA test. It is believed that transformation in the T/Dtu mixture reflects a specific immunological phenomenon, the recognition of a new foreign antigen on isogeneic tumor cells. This has been discussed elsewhere [18]. Such reactions might not occur because of immune tolerance [21], because quantitatively or qualitatively insufficient antigen is present, because inhibitory substances are in the serum, or because of the incapability of lymphocytes to transform at all. Despite its obvious lack of reproducibility when a given pair of cell donors is studied repeatedly, the MLC seems to select rather reliably the same sequence of different donor pairs, e.g. AB, AC, AD when studied simultaneously on different occasions [1] reflecting the qualitative accuracy of this test despite its quantitative limitations. In another study, which is contributory to the MLTC described herein [17] we found that erroneous interpretation of response can be diminished by testing the stimulating : responding cells at different ratios. If we had tested the lymphocyte : tumor cell ratio at only 2 : 1 in figure 2, we would have found no stimulation of T lymphocytes by D tumor cells, a completely erroneous conclusion. Reports in the literature of failure of homologous and heterologous cells other than lymphocytes to act as mitogens [9, 21] might be explained by the narrow range of ratios tested. At least malignant melanoma cells must be added to the list of cellular mitogens.

In order to obtain a pure 'one way' stimulation in the MLC it was necessary to inhibit DNA synthesis with Mitomycin C. This is unnecessary in the MLTC. Tumor cells show no significant DNA syn-

thesis under these culture conditions after 5 days, as reported herein and as shown in pilot studies. Thus no artificial management of the 'antigenic' tumor cells is necessary, which diminishes the risk of alteration or loss of surface antigens.

Possible further applications of the MLTC in the study of immunity to cancer are apparent. The transformation of T lymphocytes by T tumor cells four and one half year after operation made it likely that specifically sensitized lymphocytes circulate for long periods of time. It remains to be demonstrated whether, after therapy, the MLTC becomes positive against stored autochthonous tumor. The major advantage of the FDA test as an assay for the cytotoxic action of immune cells on histoincompatible target cells is its simple technique and its sensitivity providing positive results within a few hours. As other studies indicated [23] the intracellular retention of Fluorescein depends on the integrity of the cell membrane. Thus the fluorescein release from cells after few hours in culture suggests a membrane damage in the early phase of the reaction.

Summary

The major techniques currently used to show tumor immunity in man are summarized. Two new techniques to study cell mediated tumor specific immunity are presented. (1) The mixed lymphocyte tumor cell culture (MLTC). Tumor cells were titrated as 'antigen' in a dilution series against a given number of peripheral blood lymphocytes. The presence of foreign histocompatibility antigens is indicated by lymphocyte transformation. (2) The Fluoresceindiacetate (FDA) test. The disappearance of intracellular fluorescein from tumor target cells is a result of the cytotoxic activity of immune lymphocytes.

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The Effect of Packed Cell Volume, Hemoglobin Content and Red Cell Count on Whole Blood Viscosity

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Whole blood viscosity is a function of the amount and quality of suspended particles, such as erythrocytes, and the various plasma proteins and of the interaction between these components. The role of the hematocrit as the major determinant of viscosity has been stressed [1] It has also been shown that alterations in the shape and size of the red cells such as spherocytosis [2, 3] sickling [4, 5] microcytosis and macrocytosis [6] as well as changes in their internal rigidity [7] produced profound alterations in viscosity Most attempts to correlate viscosity with erythrocytes have been based on hematocrit values [1, 8]. Abnormally high values of hematocrit, such as found in polycythemia, have been recognized as one of the most common clinical conditions leading to excessive blood viscosity Therapeutic programmes aimed at reducing blood viscosity in polycythemia have usually been evaluated by determination of changes in hematocrit [9] However, venesections, the most common form of therapy in polycythemia, produce not only a reduction of hematocrit, but as a result of iron deficiency a microcytic hypochromic blood picture, revealed by a disproportionately high red cell count and low hemoglobin content In view of the changes in viscosity induced by spherocytosis and sickling, it could be presumed that erythrocytes with abnormal size and hemoglobin content might influence blood viscosity in a way different from normal erythrocytes at identical hematocrit values Accordingly, we have studied the effect on whole blood viscosity of all three erythroid parameters, namely, *packed cell volume, hemoglobin content* and *red cell count*, singly or in combination

Material and Methods

123 blood samples from 72 subjects were examined. There were 48 males and 24 females, and their ages ranged from 22 to 76 years. The 72 subjects included 31 normal laboratory and medical personnel, 33 polycythemic patients at various phases of treatment by venesection, and 8 patients with iron deficiency anemia.

Venous blood was collected by disposable plastic syringes over dry heparin (5 IU per ml of blood). Viscosity was measured at 37 °C in a Brookfield Cone and Plate Micro-Viscometer Model LVT, fitted with a Type N constant temperature bath (Brookfield Engineering Laboratories Inc., Stoughton, Mass.). The viscometer was calibrated daily by means of a silicone oil of 10.1 centipoise. Readings were taken at 5 shear rates: 11.5, 23, 46, 115 and 230 sec^{-1}. Each determination was repeated 3 times. Red cell counts and hemoglobin and hematocrit determinations were performed in duplicate on the same samples. Red cells were counted in a Model B Coulter Electronic Counter (Coulter Electronics, St. Hialeah, Florida) [10]. The hemoglobin concentration was determined as cyanmethemoglobin [11]. The standard of hemoglobin-cyanide solution 57.0 ± 0.15 g/100 ml supplied by the International Committee for Standardization in Hematology (ICSH) [12]. Hematocrit determinations were performed in a Clay Adams micro-hematocrit centrifuge at 12 000 g for 5 min [13].

Fibrinogen was determined by the method described by Ratnoff and Menzie [14] and total serum proteins by the standard biuret method.

Results

The mean values of viscosity at shear rates varying from 230 to 11.5 sec^{-1} and the mean hemoglobin concentration, hematocrit, red blood cell count, fibrinogen content and total serum protein are shown in table I, together with the standard errors.

Since the aim of the study was to analyse the influence of red cell parameters on whole blood viscosity, 6 samples with a fibrinogen level

Table I. Viscosity, red cell parameters, fibrinogen and serum protein of 123 blood samples

		Mean	SE
Viscosity (cP) at shear rate sec^{-1}			
	230	4.[illegible]3	0.0[illegible]1
	115	5.3[illegible]	0.0[illegible]2
	46	6.29	0.10[illegible]
	23	7.31	0.128
	11.5	9.0[illegible]	0.18[illegible]
Hemoglobin, g/100 ml		15.[illegible]3	0.210
Hematocrit, %		45.26	0.37[illegible]
Red cell count, × 10^6/mm³		3.4[illegible]	0.0[illegible]7
Plasma fibrinogen, mg/100 ml		2[illegible]3	5.22
Total serum protein, g/100 ml		6.47	0.049

above 400 mg% were excluded from further analysis in order to avoid drastic variations in the plasma protein pattern.

The remaining 117 blood samples were further subdivided according to their mean corpuscular volume. Taking the arbitrary value of 85 μm^3 as the lower limit of normal, 92 of the samples were defined as normocytic whereas the remaining 25 constituted the microcytic group. The difference between the normocytic and microcytic groups is shown in table II, by comparison of the mean hemoglobin and hematocrit values and red cell counts, and their standard errors. It is evident that for a given number of red cells, the hematocrit and hemoglobin values are lower in the microcytic group and that the standard error of all 3 parameters is higher in the microcytic group than in the normocytic group.

Figures 1–3, demonstrate the relationship between viscosity at shear rate 11.5 sec^{-1}, and the hemoglobin concentration, hematocrit and red cell counts respectively, in both the normocytic and microcytic blood samples. It can be seen that the relation of viscosity to each of the three erythroid parameters is not linear, the distribution of the normocytic and the microcytic groups is not identical, and the difference between the two groups varies in degree and direction, depending on whether

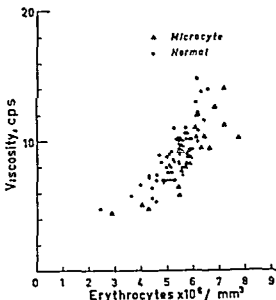


Fig. 1. Whole blood viscosity determined at shear rate 11.5 sec^{-1} related to red cell count.

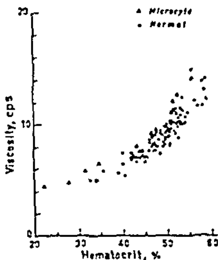


Fig. 2 Whole blood viscosity determined at shear rate 11.5 sec⁻¹ related to hematocrit.

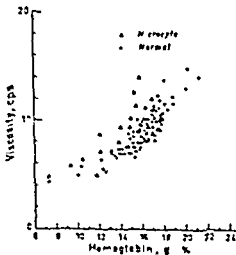


Fig. 3 Whole blood viscosity determined at shear rate 11.5 sec⁻¹ related to hemoglobin.

Table II Hemoglobin, hematocrit and red cell counts of the normocytic and microcytic groups

Group	Hemoglobin g%		Hematocrit %		Red cell count $\times 10^6/mm^3$	
	Mean	SE	Mean	SE	Mean	SE
Normocytic	15.94	0.200	49.02	0.451	5.37	0.063
Microcytic	14.78	0.627	45.46	1.742	5.79	0.200

the viscosity is expressed as a function of the hematocrit, the hemoglobin or the red cell count.

With the above considerations in mind, the 3 red cell parameters and the viscosity measurements obtained in the normocytic and microcytic groups were analysed separately.

Statistical Analysis

Hemoglobin, hematocrit and red cell count were designated X_1, X_2 and X_3, respectively. The following additional data were included: X^2_1, X^2_2, X^2_3, $X_1 \times X_2$, $X_1 \times X_3$ and $X_2 \times X_3$. These data were correlated to viscosity (Y) at all shear rates examined, i.e. 230, 115, 46, 23 and 11.5 sec^{-1}. The data were analysed by a stepwise regression procedure [15] and evaluated by means of a CDC 6400 Computer[1].

Stepwise regression procedure Let Y be a response variable and assume that one wishes to establish a linear relation between it and a set of predictor variables X_1, X_2, ..., X_K *(the latter need not be independent and often are, in fact, functionally related to each other)* If it is desired to present a regression equation which includes only variables with non-negligible contributions to the accuracy of prediction, a stepwise regression procedure may be used

The stepwise procedure used here begins with a simple correlation matrix and enters into regression the X variable most *highly correlated with the response* (X_1, *say*) *and finds the first order, linear regression* equation The partial correlation matrix of X_j (j - 1) and Y is then calculated, and the next variable to enter regression is that X variable whose partial correlation with the response is highest This process continues and as each variable is entered into the regression, the following values are examined

(1) R, *the multiple correlation co-efficient*, (2) The partial F-test value for the variable most recently entered, which shows whether this variable has taken up a significant amount of variation as opposed to those removed previously by variables in the regression At every stage, the variables incorporated into the model in previous stages are re-examined in the

[1] *Our thanks are due to* Dr GIORA HANOCH from the Department of Economic Sciences of the Hebrew University for programming the data analysis

following way A variable which may have been the most suitable single variable to enter at an early stage may at a later stage be superfluous because of the relationship between it and other variables now in the regression. To check this, the partial F criterion for each variable in the regression at all stages of calculation is evaluated and compared with a preselected percentage point of the appropriate F distribution. This provides an assessment of the contribution made by each variable as though it had been the most recent variable entered irrespective of its actual point of entry into the model. Any variable which does not provide a significant contribution is removed from the model. This process is continued until no more variables can be admitted to the equation and until none are rejected.

The final results of the stepwise regression analysis are presented in tables III, IV, V and VI These include the multiple correlation coefficients, standard error of prediction, the variables selected and their coefficients, standard errors, t values and mean square percent of variance Since the results were similar at all shear rates, only those obtained at the shear rate of 230 sec^{-1} (Y_1) and 11 5 sec^{-1} (Y_6) are presented

Normocytic group (tables III and IV) The multiple correlation coefficient was 0 93995 for Y_1, and 0 91728 for Y_6 At both shear rates, the highest t value and the highest mean square percent were those of $X_1 \times X_1$, i e the square value of the hemoglobin concentration

Microcytic group (tables V and VI) The multiple correlation coefficient was 0 91828 for Y_1 and 0 95821 for Y_6 In contrast to the former group, the highest t value and the highest mean square percent were those of $X_4 \times X_4$ i e the square value of the hematocrit, and not $X_1 \times X_1$ *Another difference from the normocytic group was*

Table III Regression equation of normal group at shear rate 230 sec^{-1} (Y_1)

Variable	Coefficient	Stand. error	t value	Mean square percent of variance
Constant	7.6291	1.3016	5.860	–
X_1	-0.4[illegible]	0.2840	-1.431	6.412
X_4	1.1[illegible]	0.9757	-1.209	4.5[illegible]
$X_1 \times X_1$	0.0[illegible]	0.0[illegible]	4.663	67.9[illegible]
$X_1 \times X_4$	-0.0[illegible]	0.00[illegible]	-0.932	3.010
$X_4 \times X_4$	0.0[illegible]	0.01[illegible]	1.43[illegible]	6.40[illegible]

Multiple R = 0.9[illegible] Stand. error of prediction = 0.[illegible]
Order of entrance or deletion of the variables $X_1 \times X_1$, X_4, $X_1 \times X_4$, X_1, $X_4 \times X_4$, X_4
Variables deleted for low tolerance $X_4 \times X_6$, $X_4 \times X_6$, $X_1 \times X_6$
Variable not entered because of low correlation coefficient X_6

Table IV. Regression equation of normal group at shear rate 11.5 sec^{-1} (Y_1)

Variable	Coefficient	Stand error	t value	Mean square percent of variance
Constant	11 5328	3 9222	2 940	–
X_2	–0 7079	0 8557	–0.827	9 264
X_3	–2 1692	2 7984	–0 775	8 134
$X_1 \times X_1$	0 0387	0 0193	2 005	54 435
$X_1 \times X_2$	–0 0045	0 0199	–0 227	0 698
$X_3 \times X_3$	0.531	0 0573	0 926	11 609

Multiple R = 0 91728, stand error of prediction = 0 78874
Order of entrance (or deletion) of the variables $X_1 \times X_3, X_1, X_2 \times X_1 \times X_2$ $X_3 \times X_3$
Variables deleted for low tolerance $X_3 \times X_3, X_2 \times X_2, X_1 \times X_3$
Variable not entered because of low correlation coefficient X_3

Table V. Regression equation of microcytic group at shear rate 230 sec^{-1} (Y_1)

Variable	Coefficient	Stand error	t-value	Mean square percent of variance
Constant	3.2804	1 4628	2.243	–
X_1	0 8145	0 5016	1 624	7 038
X_2	–0 6372	0 2520	–2.528	17 065
X_3	2.3888	0 8079	2 257	23 335
$X_2 \times X_2$	0 0092	0 0026	3 565	33 936
$X_1 \times X_3$	–0 1418	0 0825	–1 719	7 887
$X_2 \times X_3$	–0 0103	0 0207	–0 498	0 662

Multiple R = 0 94828, stand error of prediction = 0 30659
Order of entrance (or deletion) of the variables $X_2 \times X_2, X_2$ $X_3, X_2 \times X_3, X_1 \times X_3$, X_1
Variables deleted for low tolerance $X_3 \times X_3, X_1 \times X_2$
Variable not entered because of low correlation coefficient $X_1 \times X_1$

the relatively higher contribution of factors other than $X_2 \times X_2$, as revealed by their mean square percent, in the microcytic group.

The regression equation of table VI was used for evaluation of the effect on viscosity of variations in mean corpuscular volume, and mean corpuscular hemoglobin concentration at a fixed value of hematocrit. Since 45 5% was the mean hematocrit of this group, all calculations were related to this value. A three-dimensional model *describing the* effect of variations in red cell count and hemoglobin concentration on viscosity at hematocrit 45.5% is depicted in figure 4. As revealed by

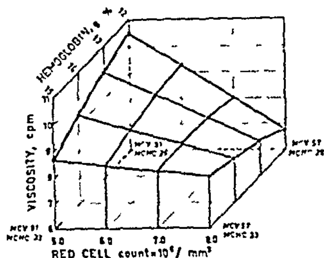


Fig. 4. Three-dimensional model describing the effect of variations in red cell count and hemoglobin on viscosity at a fixed hematocrit of 45.5%. *MCV* mean corpuscular volume (μm³); *MCHC* mean corpuscular hemoglobin concentration (%)

Table 11. Regression equation of microcytic group at shear rate 11.5 sec⁻¹ (Y)

Variable	Coefficient	Stand. error	t value	Mean square percent of variance
Constant	7.9[illegible]	3.1355	2.550	–
[illegible]	-0.9[illegible]1	0.5[illegible]1	1.[illegible]7	19.411
[illegible]	-0.0[illegible]4	0.0431	1.045	6.912
[illegible]	0.013[illegible]	0.0051	2.5[illegible]	41.53[illegible]
[illegible]	0.3[illegible]	0.2[illegible]	1.511	10.207
[illegible]	-0.11[illegible]	0.0[illegible]3	-1.437	13.331

Multiple R = 0.[illegible]; stand. error of prediction = 0.[illegible]
Order of entrance or deletion of the variables: [illegible]
Variables deleted for low tolerance: [illegible]
Variables not entered because of low correlation coefficient: [illegible]

this diagram, decreasing hemoglobin concentration tends to increase viscosity, whereas an increase in the number of red cells reduces viscosity. Since the hematocrit in this model is constant, the above statement means that a decrease in mean corpuscular hemoglobin

Table VII. Constant (a), slope (b), and correlation coefficient (r) for the expression of viscosity as $Y = a + b\ Z$[1] at different shear rates

Shear rate sec^{-1}	a	b	r
230	2.5413	0.0041	0.9256
115	2.6584	0.0047	0.9134
46	2.8559	0.0062	0.9115
23	2.6896	0.0086	0.8978
11,5	2.7232	0.0117	0.9128

[1] $Z = \frac{X_2^4}{X_1 \times X_3}$
where x_1 = hemoglobin (g/100), x_2 = hematocrit (%), and x_3 = red cell count ($\times 10^6$/mm^3)

concentration $\left(MCHC = \frac{\text{hemoglobin}}{\text{hematocrit}}\right)$ increases viscosity, and a reduction in mean corpuscular volume $\left(MCV = \frac{\text{hematocrit}}{\text{red cell count}}\right)$ decreases viscosity.

Taking into consideration the importance of X_2^2, i.e. the square value of hematocrit in the regression equation of the microcytic group, viscosity could be related directly to $\frac{X_2}{X_3}$ (MCV) and X_2^2 and inversely related to $\frac{X_1}{X_2}$ (MCHC), or

$$(Z) = X_2^2 \times \frac{X_2}{X_3} \times \frac{X_2}{X_1} = \frac{X_2^4}{X_1 \times X_3}$$

and viscosity (Y) = a + b × Z. The constant (a), slope (b) and correlation coefficient (r) have been calculated separately for each shear rate, as presented in table VII.

Discussion

The viscosity of whole blood is a joint function of soluble plasma proteins and of solid particles suspended in it, and the interaction between these elements. Although much information has been gained by the earlier technics of capillary viscosimetry, a major defect of these procedures was the poorly defined shear rate. Since blood is a non-

Newtonian fluid its viscosity varies with changes in shear rate, and the measurement of viscosity at uncontrolled shear rates is meaningless. A major advance in viscosimetry has been the introduction of devices such as the cone plate type viscosimeter used in this study, in which the shear rate is accurately controlled and is uniform throughout the whole sample tested. Although the Wells-Brookfield viscosimeter used by us has a much wider range of shear rates, we preferred to limit our observations to the range of 11.5 and 230 sec^{-1}, i.e. a range which represents the *in vivo* shear rates of blood prevailing in the aorta down the medium arterioles [16].

Using the same technique, BEGG and HEARNS [1] clearly demonstrated that the hematocrit value exerts a marked influence on whole blood viscosity, while fibrinogen and serum proteins have only a limited effect. A similar conclusion was reached at by PIROVSKY [17]. While there is no disagreement as to the important role of the hematocrit in the determination of blood viscosity, the mathematical expression of the relation between these two parameters has been controversial. A hyperbolic relationship between blood viscosity and packed cell volume was postulated by HESS in 1911 [18], and a direct linear relationship by NYGAARD *et al.* in 1935 [19]. Although methods of measurement have been refined since then, the controversy is still unsettled. Thus, BURCH and DE PASQUALE [20] have suggested a direct linear relationship between viscosity and the hematocrit, while VIRGILIO *et al.* [21], GREGERSEN *et al.* [22] and BEGG *et al.* [1] proposed a logarithmic relation. By using the Wells Brookfield type viscosimeter, ROSENBLATT *et al.* [8] found a direct linear relation between the hematocrit and whole blood viscosity measured at shear rate 230 sec^{-1}, with a correlation co-efficient of 0.71. Transformation of blood viscosity measured at the same shear rate by BEGG *et al.* [1] into logarithmic form resulted in an increase in the correlation co-efficient to 0.82, thus proving that a curvilinear expression of the relation between viscosity and hematocrit is a more correct one.

The multiple correlation coefficients of the stepwise regression procedure presented here indicate that the mathematical expression of the relation between viscosity and erythroid data can still be improved. Thus the correlation coefficient for viscosity at shear rate 230 sec^{-1} measured by the same instrument and in identical conditions to those of ROSENBLATT *et al.* [8] and BEGG *et al.* [1] was 0.93995 for the normocytic and 0.91523 for the microcytic group.

This improvement in correlation was the result of the application of a stepwise regression procedure for the analysis of our data Through this procedure an empirical formula has been constructed which utilizes not only the data available on packed cell volume, but also the *hemoglobin content and the number of corpuscles It is not unexpected* that such additional information, when correctly used, yields an im proved correlation with viscosity, since it has already been demon strated that variations in cell shape and size such as spherocytosis [2, 3], micro or macrocytosis [6] occurring spontaneously or induced *in vitro* [5] alter blood viscosity It is evident from figure 4, which presents the effect of variations in hemoglobin content and red cell count at a fixed hematocrit value, that departure from a normal MCV and MCHC results in an increase in viscosity with advancing hypochromia and a decrease in viscosity with increasing microcytosis

On the basis of these observations a simplified formula has been derived from regression equation VI which expresses viscosity (Y) as equal to $a + b \times Z$ when a and b are constant and Z is $\frac{X_2^2}{X_1 \times X_3}$ Thus Z incorporates the square value of hematocrit (X_2^2) which is the predominant variable in equation VI as judged by its t value and mean square percent, *it is directly related to mean corpuscular* volume $\left(\frac{X_2}{X_3}\right)$ and inversely to mean corpuscular hemoglobin con centration $\left(\frac{X_1}{X_2}\right)$ The advantages of such a simplified formula are obvious While still retaining a high correlation coefficient, it ex presses the influence of variations in hemoglobin (X_1), hematocrit (X_2) and red cell count (X_3) in a way which is clear at first glance It can be seen for instance, that hypochromia and microcytosis caused by *therapeutic venesections in polycythemia influence viscosity in oppos* ing directions, and thus tend to neutralize each other, leaving the *hematocrit value an overwhelming influence on viscosity*

The relative importance of each variable within the complete regression equation has been indicated by its mean square percent In the normocytic group, the highest mean square percent was that of the square value of hemoglobin and this was true for all shear rates examined The situation was quite different in the microcytic group where the highest mean square percent was that of the square value of hematocrit and the contribution of the other factors was relatively

greater than in the normocytic group. This difference in the nature of the regression equation of the normocytic as opposed to the microcytic group is thus far unexplained. We accept the hematocrit as the most important single factor related to viscosity; this is supported by the observation that even in the normocytic group the first variable to enter the regression equation included the hematocrit. The fact that hemoglobin, and not the hematocrit, became the predominant factor in the final equation might reflect the greater reliability and excellent standardization of hemoglobin determination, as compared to the other measurements. This point, however, requires further clarification.

Fibrinogen levels and the total serum protein determined in all samples included in this study, have until now been utilized only for the exclusion of samples with extreme variations, since the purpose of the present study was limited to the analysis of the influence of red cell parameters on viscosity. A project aimed at the systematic evaluation of plasma factors and their influence on whole blood viscosity is already in progress.

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Summary

The viscosity of 123 blood samples from 72 subjects was determined in a cone and plate microviscometer at shear rates ranging from 11.5 to 230 sec^{-1}. Comparison of samples of normocytic and microcytic blood revealed that the most important single factors related to viscosity were the square value of the hematocrit in the microcytic group and the square value of the hemoglobin in the normocytic group. The regression equation of the microcytic group revealed that at a fixed hematocrit value, progressive hypochromia resulted in an increase in viscosity whereas increasing microcytosis led to a decrease in viscosity. On the basis of these findings a simplified formula has been suggested, which expresses viscosity as the product of the square value of hematocrit, the mean corpuscular volume, and the inverse of mean corpuscular hemoglobin concentration.

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Leucocytosis Following Administration of Cryoprecipitate

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Concentrates of human anti haemophilic globulin (factor VIII), as prepared by the cold precipitation technique of Pool and Shannon [9] are widely used in the treatment of haemophilia. The monitoring of this form of therapy is desirable in order to ensure that haemostatic levels are achieved, and that the potency of the concentrate is maintained. Whilst monitoring the treatment of one such patient, a substantial increase in the total leucocyte count over pre-treatment values was observed by one of us (J.F.). Thereafter, similar post cryoprecipitate infusion leucocytoses were noted in other haemophiliac patients. This communication reports the preliminary investigation of this finding.

Materials and Methods

Twenty-three patients were studied. One group was composed of 17 patients suffering from a hereditary bleeding diathesis. 13 had moderate to severe haemophilia, 2 had Christmas disease, the remaining 2 had von Willebrand's disease. The second group was made up of 6 patients who were not suffering from any haemorrhagic disorder. They were surgical patients of comparable age and sex who were about to receive a blood transfusion and who consented to participate in the investigation.

Therapeutic quantities of cryoprecipitate (the pooled activity from 4–12 blood donations) were administered to the 13 haemophiliac patients on 42 occasions. Similar quantities were given to the patients with Christmas disease and von Willebrand's disease. The non haemorrhagic patients were given between 30 to 50 ml of cryoprecipitate, the pooled activity of not more than 4 donations. Comparative quantities of untreated plasma were administered to patients of both groups. The plasma used was between 24 and 48 h old.

Differential, visual and electronic leucocyte counts were carried out before and after all infusions. The post infusion counts were performed at 15 and 30 min, and every 2 h thereafter for a 24 h period. Differential counts were performed by 2 independent observers. Changes in pulse rate and body temperature were also recorded during the period of investigation.

Leucocyte counts The visual counts were carried out by a standard method as described by DACIE and LEWIS [5] The electronic counts were carried out on the Model 'A' coulter counter, with a 70μ orifice tube The factor VIII assays were estimated by the 2 stage method according to that of BIGGS and MACFARLANE [3]

Results

In all the patients suffering from a haemorrhagic disorder the administration of cryoprecipitate was followed by a rise in the total leucocyte count. The leucocytosis was characterised by an increase in the number of mature neutrophils, (fig 1) This ranged from 470 to 10,900/mm³, with a mean of 4,640/mm³. The absolute numbers of lymphocytes, monocytes, eosinophils and basophils did not alter significantly, nor did the platelet count change The pulse rate and body

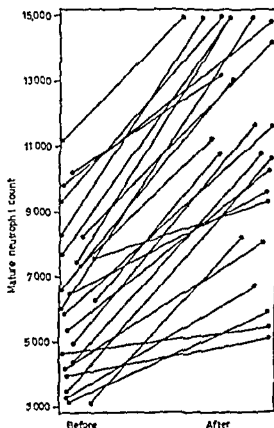


Fig 1 The neutrophil counts before and 30 min after cryoprecipitate infusions

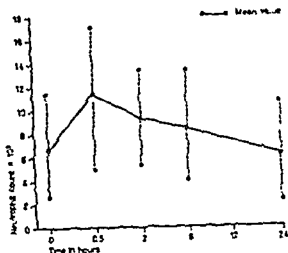


Fig. 2. Changes in neutrophil count during the 24 h post cryoprecipitate infusion.

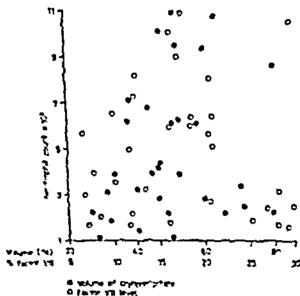


Fig. 3. The [illegible] of cryoprecipitate are given and the corresponding factor VIII attained, correlated [illegible] the neutrophil counts.

temperature showed little variation during the 24-h post infusion period. The maximum response occurred 30 min after completion of the cryoprecipitate treatment. The leucocyte count returned to pre-treatment levels during the following 24 h (fig. 2).

In the non-haemorrhagic group, 2 of the patients failed to exhibit a leucocytosis. The remainder produced a mean rise of 592 neutrophils/mm^3, with a maximum increase of 1,300/mm^3.

The infusion of untreated plasma did not alter the neutrophil count significantly in any of the patients studied.

The volumes of cryoprecipitate given, and the levels of factor VIII attained were correlated with the respective neutrophil counts (fig. 3). No relationship was demonstrated between any of the measurements.

Discussion

The homeostatic mechanism controlling the number of circulating leucocytes is not yet fully understood. The number of circulating granulocytes may be increased by a multiplicity of factors, namely exercise [1], nor-adrenaline [2], etiocholanolone [8, 10], glucocorticoids [2, 6] and bacterial toxins [4]. It has been shown that the rise induced by exercise and nor-adrenaline [1, 2] occurs within minutes, as a result of redistribution of granulocytes from the marginating to the circulating vascular pool, whereas glucocorticoids, etiocholanolone and bacterial toxins [2, 4] induce a leucocytosis by increasing the number of granulocytes released from the bone marrow. A latent period of 4–6 h elapses before this leucocytosis becomes apparent.

The rapid response evoked by the cryoprecipitate infusion appears similar to that produced by nor-adrenaline and exercise, suggesting that it too may be a pseudo-leucocytosis caused by cell shifts within vascular compartments. However, the precise nature of the leucocytosis remains obscure. It did not occur in response to untreated plasma. Therefore, the stimulus would appear to be within the cryoprecipitate.

The leucocytosis may be part of a general response to an unknown component contained in the concentrate, because, although all the recipients did not respond in an identical manner, only 2 of the 23 patients failed to exhibit some degree of neutrophilia and they belonged to the 'non haemorrhagic' group. There appeared to be a significant difference between the response of this group, and that of

the hereditary 'haemorrhagic' patients The difference could not be related to the levels of the 2 specific coagulation factors concerned, as both the factor VIII and factor IX deficient patients responded in a like manner Furthermore, the concentration of factor IX in cryoprecipitate is not comparable to that of anti haemophilic globulin

It is possible that the 'haemorrhagic' patients were conditioned to respond in the manner described It may be, that their preceding bleeding episode in some way altered the normal mechanism for controlling leucocyte migration and rendered it more sensitive KATZ *et al* [7] have shown from animal experiments that humoral agents, which produce an increase in circulating leucocytes, are evoked either by removal, or by destruction of mature leucocytes. Such destruction of cells is likely to occur during the production of cryoprecipitate resulting in the production of a plasma factor, which is capable of inducing the described leucocytosis in a suitable recipient

Further studies are to be undertaken to relate the occurrence and intensity of the neutrophilia to the time of any preceding bleeding episode, also further biochemical investigations will be carried out in an attempt to identify the 'leucocytosis promoting factor'.

Summary

A significant neutrophil leucocytosis was demonstrated in patients, suffering from a hereditary bleeding diathesis, following cryoprecipitate treatment

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Trypsin Activity of Haemophilic Blood

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In a study on the trypsin activity of various biological fluids an unexpected finding was the low activity of plasma and serum of haemophiliacs as compared with normals.

Materials and Methods

Samples of plasma and serum were obtained, as described by BIGGS and MACFARLANE [1] from 30 normals and 30 patients with severe haemophilia A. The samples were kept in ice until the performance of the test.

The trypsin activity was determined by [illegible] method [3]. To a mixture of 0.5 ml of plasma or serum and 0.5 ml of phosphate buffer (91.5 ml M/15 Na_2HPO_4 and 8.5 ml M/15 KH_2PO_4), one ml of 0.1 M benzoyl-l-arginine amide hydrochloride was added to the same phosphate buffer. The mixture was then agitated in a shaker for one hour. An 0.2 ml aliquot of the mixture was then analysed for ammonia by the CONWAY microdiffusion technique. The trypsin present in the original sample is proportional to the amount of ammonia produced which was titrated by an 0.001 N HCl solution. Crystalline lyophilized trypsin [illegible] was used as a standard. The results are expressed in units of trypsin activity per ml, 100 units being arbitrarily taken as the equivalent to 100 µmoles of trypsin.

Normal values: Plasma [illegible] units, mean 12[illegible]
Serum [illegible] units, mean 150

Results

As the figure shows, samples of plasma and serum of patients suffering from severe haemophilia A exhibited a very low trypsin activity.

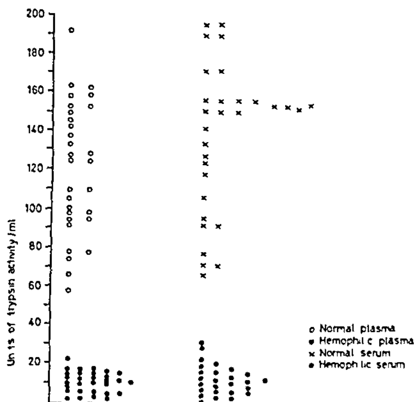


Fig 1 Trypsin activity of haemophilic plasma and serum as compared to normal

Discussion

The present study seems to indicate that haemophilic plasma and serum possess low trypsin activity as compared with normal The clot accelerating effect of trypsin is well documented The reader is referred to the publications of FERGUSON *et al* [2], STORMORKEN [5] and PECHET and ALEXANDER [4] In spite of the extensive literature there exists no general agreement on the mechanism of the trypsin effect on blood coagulation A search of the available literature failed to find reports on the trypsin activity of haemophilic plasma or serum At present we are unable to interpret the findings Further work is in progress

Summary

In haemophilia the trypsin activity of plasma and serum is low as compared with normal.

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Congenital Hypoprothrombinemia

Case Report

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Congenital hypoprothrombinemia seems to be the rarest coagulation disorder. Up to now only 14 proven cases have been described in the world literature [2, 4, 8, 12, 13, 21, 22, 30, 32, 39] Recently 2 patients with an abnormal prothrombin molecule have been reported [36].

The object of the present paper is to describe another patient who showed a coagulation defect consistent with the diagnosis of true congenital hypoprothrombinemia This is the third patient with congenital hypoprothrombinemia seen in Italy The other two patients were studied by us in 1968 and 1969, respectively [12, 13]

Case Report

The propositus is a 45-year-old white male He is a first cousin of another patient with congenital hypoprothrombinemia already described by us [12] The patient was referred to us for an evaluation of his bleeding tendency in October 1969 His parents were not consanguineous but came from the same small village and were asymptomatic A sister was instead referred to as a bleeder but could not be examined The first bleeding manifestation occurred at the age of 5 when the patient had to undergo surgery for the repair of a right inguinal hernia Massive bleeding occurred immediately after surgery and resulted in a huge hematoma of the inguinal area The patient was transfused with 1 unit of whole blood and treated with non specific coagulant therapy At the age of 6 he started complaining of occasional epistaxis which have occurred throughtout his life At the age of 11 the patient was admitted to a local hospital because of fever nausea and pain in the right hip area The diagnosis of osteomyelitis of the head of the femur was formulated and the patient was treated for several months with bedrest and unidentified medications There was no intra articular bleeding Since that time the patient has been complaining of mild limping and of some limitation in the abduction movements of the right leg At the present time the condi

tion is referred to radiologically as 'coxarthrosis of the right hip'. At the age of 14 the patient presented profuse bleeding after his first tooth extraction. The bleeding was so persistent that the patient had to be admitted to a local hospital where he was treated with topic and parenteral non-specific coagulant therapy.

A few years later, the second tooth extraction was again followed by excessive bleeding. At the age of 21 the propositus presented a laceration of the tongue secondary to an accidental fall. Bleeding was very severe and the patient was hospitalized. At the age of 33 the propositus underwent surgery for a left inguinal hernia repair. Bleeding was massive and the patient had to be transfused with 2 units of whole blood.

In the following years the patient underwent 5 other tooth extractions which were always followed by excessive bleeding in spite of non-specific coagulant therapy given prophylactically.

Two years ago, at the age of 4[illegible], the patient presented a hemarthrosis of the right knee after an accidental fall from the bicycle. The swelling and the limitations of function were so marked that the patient was bed-ridden for about 25 days. He was given no transfusions but only non-specific coagulant therapy. There were no sequels. The bleeding manifestations seem to have subsided during the past 10 years. Spontaneous bleeding has always been mild and consisted of epistaxis, easy bruising and bleeding from the gums. At the time of study there were no bleeding manifestations.

Material and Methods

Plastic syringes (Pharmaseal) and gauge No. 19 siliconized needles.

3.8% sodium citrate. Calcium chloride 0.025 M. Michaelis buffer solution at pH 7.3. Imidazole buffer solution at pH 7.3. Veronal buffer solution at pH 7.3.

Tissue whole thromboplastin: Simplastin (Warner-Chilcott) or Rabbit brain thromboplastin (Ortho). Activated partial thromboplastin: Cephaloplastin (Dade). Thrombin topical (Topostasin, Roche; Thrombin, Behringwerke). Bovine Fibrinogen (Armour). Chemical [illegible] plasma (Dade). Prothrombin free beef plasma (Hyland).

Congenital factor XII deficient plasma was obtained through the cooperation of the [illegible] Department of Clinical Pathology, Mayo Clinic, Rochester, Minn., USA.

Fresh factor VIII, factor IX and factor XII deficient plasma was obtained from individuals with congenital severe deficiencies of these factors. Frozen factor X deficient plasma was obtained from a patient with severe factor X deficiency.

Celite [illegible] grade [illegible] obtained from Sigma Laboratories was used. [illegible]

Russell viper venom (Burroughs Wellcome). The dried preparation was reconstituted with the [illegible] supplied by the manufacturer to obtain a 1:1000 solution.

Tiger snake venom [illegible] by the Koch-Light Laboratories. The powder supplied by the manufacturer was reconstituted with Michaelis buffer to obtain a solution containing 100 µg per ml.

[illegible] preparation was obtained from Diagnostic Reagents Laboratories. Standard normal plasma was obtained [illegible] of citrated plasma with [illegible] mg of barium sulphate [illegible]. The [illegible] was then separated by centrifuging at 3000 [illegible]. The [illegible] of the standard normal plasma [illegible]. Factor II, factor X, factor VII, factor X [illegible] substrates [illegible] by the Sigma Laboratories were used. [illegible] 2-stage reagent and [illegible] was obtained from [illegible] Laboratories. Factor II and factor VII [illegible] substrate were [illegible] by Sigma Laboratories.

Plasma was obtained by centrifuging at 5° C 1 10 citrated blood for 10 min at 2 000 rpm unless otherwise specified All tests were carried out in normal glassware unless otherwise specified The glass clotting time was performed according to the method of LEE and WHITE [25] Platelets were counted according to the method of BRECHER and CRONKITE [5] Clot retraction and the tourniquet test were carried out by routine procedures [6, 28] Bleeding time was determined by the DUKE's method [28] Prothrombin time was determined by the QUICK's method [31] The prothrombin proconvertin test was performed according to the method of OWREN and AAS [29] modified by WARE and STRAGNELL [40] The factor II + factor X complex was evaluated according to a modification of the method of HJORT et al [18] Prothrombin free beef plasma was incubated with equal parts of a Russell viper venom-cephalin mixture and a 1 10 dilution of the test plasma in Michaelis buffer After a 5 min incubation period 0 1 ml of a 0 025 M $CaCl_2$ solution was added and the clotting time measured *Factor II activity* using as *thromboplastin the Tiger* venom solution, was evaluated according to a modification of the method proposed by JOBIN and ESNOUF [20] 0 1 ml of prothrombin free beef plasma was placed in a test tube together with 0 1 ml of a 1 10 dilution of the test plasma 0 1 ml of a 1 2 mixture of the Tiger venom solution in an activated Cephalin preparation was then added immediately followed by 0 1 ml of $CaCl_2$ Prothrombin-coagulase activity was assayed according to the method proposed by SOULIER et al [37, 38] using reagents kindly supplied by the Stago Laboratories

Factor II activity alone according to the HJORT s method was determined by incubating 0 1 ml of the S gma factor II and factor VII deficient substrate with 0 1 ml of a 1 10 dilution of the patient plasma and with 0 1 ml of a Stypven Cephalin mixture After a 30 second incubation period 0 1 ml of the usual $CaCl_2$ solution was added and the clotting time measured

The two stage prothrombin test was carried out according to the method of WARE and SEEGERS [41] using the Bacto reagents The Stypven clotting time was obtained measuring the clotting time of a mixture of the patient's plasma and 0 1 ml of a 1 10 000 dilution of Wellcome Russell viper venom on addition of 0 1 ml of 0 025 M $CaCl_2$ The platelet rich plasma for the Stypven clotting time was obtained by centrifuging non-contacted 1 10 citra [illegible]

plas [illegible]
usual $CaCl_2$ solution Factor II, factor X and the factor VII + factor X complex were determined by the methods of OWREN modified by LEWIS et al [26] Factor VII activity alone was evaluated using as substrate equal parts of lyophilized factor VII deficient plasma and adsorbed normal plasma *Factor X activity was determined according to the Stypven* Cephalin method [1 9] Charcoal filtered ox plasma was used as substrate A normal reference curve for factor II factor X, factor VII + factor X complex factor VII and factor X assays *was constructed using serial dilutions of normal plasma in Michaelis buffer* The factor II + factor VII + factor X complex and the Thrombotest were carried out on 1 10 citrated plasma in normal glassware The 'K test was performed by mixing a 1 10 Michaelis buffer dilution of the test plasma with adsorbed normal plasma and then by adding to such mixture a trypsin and calcium solution (Stago Factor VIII and factor IX were determined according to the method of LANGDELL et al [24] modified by HARDISTY and MC PHERSON [15] The PTA level was determined according to the method proposed by HOROWITZ et al [19] Hageman factor activity was determined by a one stage method using as substrate non-contacted plasma of a patient with Hageman trait [11, 33] This latter assay was carried out in siliconized glassware

Antiprothrombin activity was evaluated by assaying the prothrombin level of 1 2 mixture of the propositus plasma and normal plasma at serial incubation times

The prothrombin consumption test was carried out on serum obtained from blood which had been allowed to stand at 37 C for 2 hours [6 31] The partial thromboplastin

time was evaluated by the method of [illegible] *et al.* [24] using an activated partial thromboplastin. The thromboplastin screening test was performed according to the method of [illegible] and [illegible] [17] using an activated partial thromboplastin. The thromboplastin generation test was carried out according to the Oxford method using as platelet substitute a non-activated cephalin preparation [3]. Blood fibrinogen was evaluated according to a modification of the Quick's method [10, 31].

Fibrinolysis was assayed by means of an euglobulin method [7]. Thrombin time was evaluated by measuring the clotting time of a 1:2 mixture of citrated plasma and distilled water on addition of 6 NIH units of thrombin.

The thromboelastogram was obtained on a Hellige 2001 D apparatus using 1:5 citrated platelet rich plasma.

Immunodiffusion studies were carried out using an anti-human prothrombin serum kindly supplied by Dr. [illegible] of Behringwerke Laboratories.

The immunological assay of prothrombin was obtained according to the method proposed by [illegible] [27] using anti-human prothrombin serum.

Factor II activity in the propositus' relatives was assayed by the classical one stage method.

Routine liver function tests were carried out by accepted hospital procedures.

Results

The results of the coagulation tests are reported in table I. The prolonged prothrombin time was corrected by normal plasma and aged plasma but not by serum or adsorbed plasma (table II). Furthermore the plasma of our propositus corrected the prothrombin time of patients with factor V, factor VII, factor X deficiencies and with the

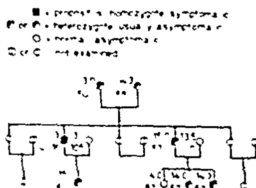


Fig. 1. [illegible] pedigree. The [illegible] on the left side of each symbol [illegible] the lower numbers refer to the prothrombin level in %.

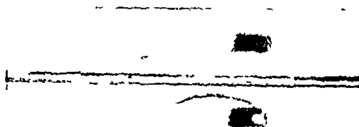


Fig 2 Immunoelectrophoretic pattern with anti human prothrombin serum The lower precipitate refers to a normal plasma The upper one to the propositus' plasma the line of precipitation in this case is barely visible



Fig 3 Prothrombin assay according to the technique of MANCINI [27] 1 = undiluted normal plasma 2 = 1 2 diluted normal plasma (50%) 3 = 1 4 diluted normal plasma (25%) 5 = undiluted plasma of the propositus The wells 4, 6, 7 and 8 refer to 4 other patients with congenital hypoprothrombinemia Two of these latter patients have been reported [12, 13], the other two are unpublished

abnormal factor X (factor X Friuli) hemorrhagic condition (table III) [14].

The plasma of another patient with proven congenital hypoprothrombinemia failed to correct the defect

The prothrombin level as determined by any of the several procedures used was definitely decreased. The levels ranged from 12% in the 2-stage method to 16% in the one-stage method (table IV). Immunologically a level of 14% was found (fig 2 and 3). The prothrombin-proconvertin test and the prothrombin-factor X complex were definitely prolonged or decreased

No inhibition of prothrombin was detected on incubation at 37°C of mixtures of the patient's plasma and normal plasma (table V) The Stypven clotting time and the Stypven-Cephalin time were slightly prolonged. The glass clotting time and the recalcification time were at the upper normal limits

Table I. Coagulation tests

	Propositus	Normal values
Platelet number	270 000	150-350 000
Tourniquet test	negative	negative
Bleeding time, min	4	2-5
Clot retraction, complete after	6 hours	12 hours
Glass clotting time, min	10	5-10
Recalcification time, sec	165	100-180
Thromboplastin screening test	70 sec in 8 min	<11 sec in 6 min
Thromboplastin generation test	28 sec in 10 min	<16 sec in 6 min
Prothrombin consumption, %	>90	>90
Partial thromboplastin time, sec	47.1	<45
Prothrombin time, sec	17.3	13
Stypven clotting time, sec	39.7	14-20
Stypven-cephalin clotting time, sec	15.3	10-14
Prothrombin-proconvertin, sec	44.0	28.5
Prothrombin factor X-complex, sec	45.3	25.5
Factor II, %	see table II	80-150
Factor V, %	87	80-130
Factor VII, %	170	80-150
Factor VIII, %	100	80-150
Factor IX, %	95	70-150
Factor X, %	90	80-150
Factor XI, %	70	60-150
Factor XII, %	110	60-150
Fibrinogen, mg%	270	270-400
Euglobulin lysis time, hours	13	10-30
Thrombin time, sec	18	18-25
Thromboelastogram: r, mm	21	10-20
k, mm	17	6-12
ma, mm	55	50-66

The partial thromboplastin time was mildly prolonged. The prothrombin consumption was normal: less than 10% of the basal plasma prothrombin was recovered in the serum 2 h after clotting.

The thromboplastin screening test showed a delayed and defective thromboplastin generation. Thromboplastin generation was shown to be defective and was corrected either by substituting the patient's serum or the patient's adsorbed plasma with normal serum or adsorbed normal plasma respectively (fig. 4). The thromboelastographic pattern was characterized by a slightly prolonged r (22 mm) and a borderline k (12 mm). The maximal amplitude on the contrary was normal (52 mm).

Table II Prothrombin time correction studies

Mixture	Results, sec
Patient plasma	17.3
0.05 ml patient plasma + 0.05 ml adsorbed normal plasma	22.6
0.05 ml patient plasma + 0.05 ml aged normal plasma	14.4
0.05 ml patient plasma + 0.05 ml normal serum	20.0
0.05 ml patient plasma + 0.05 ml fresh normal plasma	14.0
0.05 ml patient plasma + 0.05 ml plasma of Coumadin treated patient[1]	23.0

[1] The prothrombin time of this patient was 35.0 sec

Table III Prothrombin time cross studies with the plasmas of patients with deficiencies or abnormalities of prothrombin complex factors

Mixture	Clotting time seconds	Comment
0.05 ml patient plasma + 0.05 ml plasma of another patient with congenital hypoprothrombinemia[1]	19.5	The propositus' plasma was fresh, the plasma of the other patient was frozen
0.05 ml patient plasma + 0.05 ml plasma of a patient with parahemophilia[2]	14.6	same as above
0.05 ml patient plasma + 0.05 ml plasma of a patient with factor VII deficiency[3]	15.1	The propositus' plasma was fresh, the plasma of the other patient was lyophilized
0.05 ml patient plasma + 0.05 ml of plasma of a patient with factor X deficiency[4]	16.3	The propositus' plasma was fresh, the plasma of the other patient was frozen
0.05 ml patient plasma + 0.05 ml plasma of a patient with an abnormal factor X (factor X Friuli)[5]	14.3	The propositus' plasma was fresh, the plasma of the other patient was frozen

[1] The prothrombin time of this patient was 21.7 sec

[2] The prothrombin time of this patient was 34.2 sec.

[3] The prothrombin time of this patient was 48 sec (lyophilized plasma)

[4] The prothrombin time of this patient was 111 sec

[5] The prothrombin time of the patient with the factor X abnormality was 33.7 sec

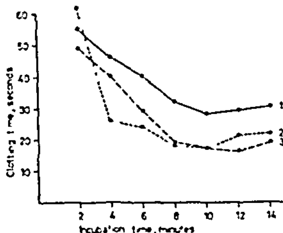


Fig 4 Thromboplastin generation test 1 = basal tracing 2 = Incubation mixture containing 0.2 ml of patient's serum and 0.2 ml of adsorbed normal plasma 3 = generation mixture containing 0.2 ml of normal serum and 0.2 ml of patient's adsorbed plasma.

Platelet morphology and function tests were all within normal limits Vascular tests were also negative

Blood fibrinogen and fibrinolytic activity, factor V, VII, VIII, IX, X, XI and XII were within normal limits

To prove beyond any doubt that we were dealing with a case of congenital deficiency the patient was given 10 mg of vitamin K_1 for 5 days and then retested No changes in the prothrombin time and in the one-stage prothrombin level were noted Routine liver function tests were within normal limits

The prothrombin time values in the relatives of our propositus were normal or slightly prolonged The factor II assay however showed that both parents, a brother and the son of our patients had moderately decreased levels and had therefore to be considered as heterozygotes (fig 1)

Discussion

Quick's criteria for true prothrombin deficiency are a prolonged one stage prothrombin time corrected by normal or aged plasma but not by serum or adsorbed plasma [32] Our patient meets fully these criteria

Table II Prothrombin level in the patient plasma as determined by different techniques

Method	Prothrombin level %	Comment
Classical one stage method [26]	16	
Iowa two-stage method [41]	12	
HJORT's method [18] (modified)	15	A Stypven-cephalin mixture is used in this method as thromboplastin
JOBIN and ESNOUF's method [20] (modified)	16	A Tiger venom (Notechis scutatus scutatus) preparation is used in this method as thromboplastin
Staphylocoagulase [38]	14	
Immunological [27]	14	

Table I Prothrombin content of 1 2 mixture of patient plasma and normal plasma as determined by the one-stage and two-stage techniques

Incubation time, hours	One-stage method %	Two-stage method %
1	58	50
5	65	48
10	55	48
24	60	55

The bleeding manifestations of our patient are typical of congenital hypoprothrombinemia epistaxes, easy bruising, bleeding from the gums, bleeding after tooth extractions or surgical procedures [12, 13, 21, 32, 39] Our case showed also a post traumatic hemarthrosis This hemophilia-like feature was noted only in about half the patients with congenital prothrombin deficiency [16, 20]

It is interesting to note the excellent correlation found among the several methods employed to assay prothrombin in our patient The good correlation found between the classical coagulation method and the immunological method clearly indicates that our patient had a true prothrombin deficiency and not a prothrombin molecule abnormality [36] The prothrombin level as determined by the two-stage method has been fairly constant in the majority of patients investigated

varying only from 8 to 14% [4, 13] This seems to be remarkable in view of the discrepancies often reported in coagulation studies

The only mild prolongation in the prothrombin time observed in our patient is in agreement with the data reported by others [14] It seems that in the presence of normal levels of activators the level of prothrombin plays a minor role in 'fixing' the duration of the prothrombin time In perfect agreement with these data is the observation that in some of our heterozygote patient the prothrombin time was perfectly normal In the heterozygote population for other prothrombin complex factors there is usually a prolongation of the prothrombin time by 1–3 seconds

The partial thromboplastin time in our case was also only slightly prolonged This observation is in agreement with the values found by Borchgrevink *et al* [4] It is likely that even in the presence of partial thromboplastin enough thrombin is formed in spite of the hypoprothrombinemia to assure the formation of an adequate clot in a normal or nearly normal time [12]

The double correction of the thromboplastin generation time is not surprising This phenomenon was seen by us in another patient with congenital hypoprothrombinemia [13] A complete correction on substitution of the patient's serum with normal serum and a partial correction on substitution of the patient's adsorbed plasma with adsorbed normal plasma were reported by Josso *et al* [21] These results are probably due to the fact that traces of prothrombin are needed in the thromboplastin generation system These traces of prothrombin are usually supplied both with the adsorbed normal plasma and with the normal serum. In a case of hypoprothrombinemia these traces of prothrombin are lacking both in the serum and in adsorbed plasma because of the lower levels existing Therefore it is clear that the substitution of either of the two patient's components with a normal equivalent will correct the defect

The data so far gathered with regard to heredity strongly suggest heterozygosity for our propositus. This is well demonstrated by the fact that both his mother and father had reduced levels of factor II consistent with heterozygosity The son and a brother of our propositus are heterozygotes too Our data suggest that congenital hypoprothrombinemia is transmitted as an autosomal incompletely recessive trait The few data available in the literature [23] notably the pedigree presented by Hart [16] are in agreement with this interpretation.

The perfectly normal factor VII, factor IX and factor X levels found in our patient with congenital hypoprothrombinemia seem to weigh against SEEGERS's theories, namely that these factors are part or subunits of the prothrombin molecule [34, 35].

Acknowledgement We wish to express our gratitude to Dr OWEN and to Dr TROBISCH for supplying us with factor VII deficient plasma and with anti human prothrombin serum, respectively

Summary

A case of congenital hypoprothrombinemia in a 46-year-old white male is presented, who complained of easy bruising, bleeding from the gums, epistaxis, bleeding after tooth extractions and post traumatic hemarthrosis.

The main laboratory features are reported The patient was probably homozygote The parents, the son and a brother had both a slightly decreased factor II level and were considered to be heterozygotes The condition seems to be transmitted as an autosomal incompletely recessive trait.

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Haemorrhagic Disease due to Hereditary Deficiency of Factor XIII

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Haemorrhagic Disease due to Hereditary Deficiency of Factor XIII

Description of a Family

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Factor XIII (fibrin stabilizing factor or F.S.F., Laki Lorand's factor, fibrinase), identified by Robbins [23] and by Laki and Lorand [14] participates in the last stages of clotting processes. This factor, activated under influence of thrombin in presence of calcium ions, acts enzymatically on primitive fibrin (the so-called soluble fibrin or fibrin s), in which fibrin monomers are aggregated only by hydrogen bonds, causing the formation of stronger peptide linkages between fibrin monomers and so transforming fibrin s into fibrin i (insoluble fibrin). Its better cohesion and resistance makes the latter, differently from the former, insoluble in solutions of monochloroacetic acid, concentrated urea etc.

In 1960 it could be ascertained that factor XIII is of primary importance in ensuring a normal haemostasis, as an almost complete deficiency in this factor in 2 brothers with a severe haemorrhagic disease clearly demonstrated [9]. About 20 more cases of congenital haemorrhagic disease due to factor XIII deficiency have been reported in the following years [12, 18, 17, 13, 11, 1, 2[illegible], 3, 15, 19, 10, 2, 5]. In this paper we shall describe 2 additional cases which occurred in the sibship of related parents, who are under our care from the time of first diagnosis in 1966.

Case Reports

Family history. Father and mother of our patients are related (fig. 1). They have always enjoyed good health and, like all the other members of the family (their sibship excluded

Table I Haemostasis studies

	Case 1	Case 2	Father	Mother	Normal values
Bleeding time [DUKE] min	3	3	2	3	<6
Tourniquet test	neg	neg	neg	neg	neg
Clotting time [LEE WHITE] min	7	6½	6	11	<12
Recalcification time [HOWELL] sec	66	77	74	90	<120
Protrombin time [QUICK] Patient/control, sec	12/12	12/12	12/12	12/12	12–14
Thromboplastin generation test	normal	normal	normal	normal	9–16 sec after 6 min
Platelets/mm³ [FONIO]	382,000	320 000	345 000	245 000	150 000–450 000
Clot retraction (visual)	normal	normal	normal	normal	
Fibrinogen [RATNOFF and MENZIE], mg%	270	280	280	265	200–400
Euglobulin lysis time [COPLEY *et al*] min	120	90	165	90	90–320
Thromboelastogram (on platelet rich plasma)					
r	6	6	7	5	5–10
k	6	5	4	7	5–8
maximal amplitude	42	19	55	43	50–60
amplitude after 2 h	5	4	48	37	

Factor XIII Activity

1 *Solubility of clot from recalcified plasma in 5 M urea* The results are reported Both patients showed quite an abnormal solubility of clots, which were completely dissolved after 60 min in case 1 and after 90 min in case 2 On the contrary the clots from the parents, as well as from 10 normal subjects, did not show any evidence of an even partial dissolution after 36 hours

2 *In vitro correction of abnormal clot dissolution with plasma from normal subjects and from patients' parents* Even minimal amounts of normal plasma could correct the abnormal solubility of patients' clots The plasma from both parents had a reduced correcting capacity (table II)

3 *The monoiodoacetate (MIA) tolerance test* demonstrated a striking difference between the plasma of patients and that of normal subjects In the patients it gave results of 8 and 12 respectively, while in normal

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Table II Solubility in 5 M urea of clot from case 2 *In vitro* correction with plasma from normal people and from parents

Dilution	Normal plasma	Mother's plasma	Father's plasma
undiluted	+ + + + 36th h	+ + + + 36th h	+ + + + 36th h
1 2	+ + + + 35th h	+ + + + 36th h	+ + + + 36th h
1 4	+ + + + 36th h	+ + + + 36th h	+ + + + 36th h
1 8	+ + + + 36th h	+ + + + 36th h	+ + + + 36th h
1 16	+ + + + 36th h	+ + + + 36th h	+ + + + 36th h
1 32	+ + + + 36th h	+ + + − 12th h	+ + + − 12th h
1 64	+ + + + 36th h	− − − − 12th h	− − − − 18th h
1 128	+ + + + 36th h	− − − − 6th h	− − − − 6th h
1 256	+ + + + 36th h	− − − − 3rd h	− − − − 4th h

+ + + + = intact clot + + + − = partially fragmented clot, + + − − = completely fragmented clot + − − − = almost completely dissolved clot − − − − = completely dissolved clot.

Table III Monoiodoacetate tolerance test [24]

		Standard test	Reduced test
Case 1		8	8
Case 2		12	9
Father (higher value of 4 determinations)		28½	
Mother (higher value of 4 determinations)		28½	
Normal people (25 subjects)	Range	31–43	
	Average	36.8	
	Standard deviation	3.8	

people it ranged from 31 to 43 In patients' parents we repeatedly recorded values lower than those of normal people (table III)

4 *In vivo correction of coagulation defect with fresh normal plasma* In case 1 the effect of plasma infusion on factor XIII activity has been studied After transfusion of 100 ml of fresh normal citrated plasma the patient's plasma became insoluble in 5 M urea, the MIA tolerance test was partially corrected, the amplitude of thromboelastogram increased (table IV) These effects gradually disappeared in about 14 days, excepted of clot indissolubility *in 5 M urea, which* persisted longer These results are in agreement with those of DUCKERT *et al* [9], IKKALA *et al* [13], LOSOWSKY and HALL [15], OTTAVIANI *et al* [19],

Table II *In vivo* correction of coagulation defect in case 1 after infusion of 100 ml fresh normal plasma

	Clot solubility in 5 M urea	MIA tolerance test		Thromboelastogram			
		Standard	Reduced	r	k	Maximal amplitude	after 2 h
Before	dissolved 1st h	8	8	5	6	47	5
After 10 min	insoluble 48th h	10	27	3	6	60	45
After 7 days	insoluble 48th h	11	16	7	7	51	26
After 14 days	insoluble 48 th h	8	11	6	9	44	11

BRITTEN [5], who could demonstrate that factor XIII has a biological half life of 4–7 days and that the correction of the coagulation defect in factor XIII deficient subjects by normal plasma lasts 15–25 days

The remarkably prolonged correction of the abnormality by small infusions of plasma offers a possibility to prevent haemorrhagic episodes Indeed our case 1 has been under periodical plasma infusions (100 ml every 14–21 days) for about 24 months During all this time she has never had any haemorrhagic manifestation, except of subcutaneous and intramuscular haematomata of left thigh (occurred after trauma in May, 1967 and in November, 1968) and of two episodes of melaena, in August, 1969 and in October, 1969, when she stayed more than 25 days without plasma infusion Case 2 refused prophylactic treatment till November 1968, when he suffered from intracranial haemorrhage with transitory partial vision loss From this time he too has been under periodical plasma infusions, with no haemorrhagic manifestations, excepted of a haematoma of right thigh and haematuria, occurred after trauma in November 1968, when he stayed 1 month without plasma infusion Till now no reduction in the effect of transfused plasma has been detected in both cases by laboratory studies

Discussion

The haemorrhagic disease of both cases is undoubtedly due to a congenital deficiency of factor XIII The clinical and laboratory picture is the same as in the few other cases reported in the literature In both cases the first bleeding episode took place soon after birth, with conspicuous umbilical haemorrhage, this particular localization

is recorded in most of the described cases There followed subcutaneous, intramuscular and dental haemorrhages, usually induced by even slight traumata In case 1 mucosal bleeding (from urinary and gastro-intestinal tract) had remarkable clinical relevance, as it was sometimes life-threatening Mucosal bleeding was reported in only few cases In both cases there occurred haemarthroses, this too an unusual fact in this disease.

It seems a typical feature of this haemorrhagic disease that abnormal bleeding usually begins 24–36 hours after the causal event This is in agreement with the abnormality of the clotting process which does not delay clot formation, but rather produces a particularly labile clot, which cannot ensure a prolonged haemostasis

Blood and plasma transfusions, as in previously reported cases, have always had a prompt effect in bleeding episodes Plasma infusions had also a good prophylactic effect in both cases

Our patients did not show defective wound healing, reported in many cases, which BECK *et al* [4] attributed to a defective growth of fibroblasts in the abnormal fibrin produced in these patients This negative finding, reported also in other cases, may be due to the precocious and frequent transfusions Besides, we must remember that BECK's experimental results have not been confirmed by OTTAVIANI *et al* [20] Malformations like those described by DUCKERT [8] in some of his cases wen also absent.

Both parents of our patients showed a partial deficiency in factor XIII activity, which could not be attributed to any known cause of reduced activity of this factor Analogous findings are reported in DUCKERT's families [8], in case 2 of JOSSO *et al* [13], and in the cases described by BARRY and DELAGE [3] and by BRITTEN [5] Different results arec reported in other families Only the father of LOSOWSKY and HALL's [15] cases 1 and 2 showed reduced activity of factor XIII Both parents have been found normal in the families described by MANDELLI [17], by OTTAVIANI *et al* [19], by MASURE [18], by BARBUI *et al* and by LOSOWSKY *et al* in their case 3 [15]

The inheritance pattern of this coagulation defect is not yet clear It is possible, as asserted by JOSSO *et al* [13] and by DUCKERT [8], that the gene responsible for defective factor XIII activity is transmitted as a partial recessive autosomal character, possibly with different expressivity and penetrance in heterozygotes It is also possible that the defect is transmitted by the X chromosome In this way it occurs

in completely affected males and females and in heterozygotes females, the latter sometimes showing detectable factor XIII deficiency according to LYON's hypothesis of X chromosome mosaicism [16]. It must also be considered that congenital deficiency of factor XIII may result from mutations, having different inheritance patterns [22]

Summary

Two cases (sister and brother) of haemorrhagic disease due to hereditary deficiency of factor XIII are described The disease became evident from first days of life with umbilical haemorrhage, and went on continuously with post traumatic bleedings In the girl mucosal bleedings from urinary and gastro-intestinal tract had particular clinical relevance In the boy an intracranial haemorrhage occurred The diagnostic importance of clot solubility and thromboelastogram is stressed Family studies showed that both parents (who are related) carried partial deficiency of factor XIII activity Prophylactic treatment has been possible with regular infusion of 100 ml of plasma every 15 days

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Diagnostisch verwertbare Veränderungen der Erythrozyten im Blutausstrich bei Paraproteinämien

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Seit mehreren Jahren sind mir färberische und physikalische Veränderungen der Blutkörperchen, besonders der Erythrozyten (E), im routinemässig behandelten Blutausstrich von Patienten mit Plasmozytom aufgefallen. Die färberischen Veränderungen sind schon makroskopisch zu erkennen. Der Blutausstrich (panoptisch mit Giemsa nach Methylalkoholfixierung gefärbt, die Pappenheim-Färbung gibt nicht an), weist eine deutliche Inhomogenität der Färbung auf. Anstelle des einheitlichen Rosa sind vielfältige Farbnuancen von rosarötlich bis rot-violett (Regenbogenfarben) vorhanden. Ausserdem ist die Gesamtoberfläche des Ausstriches nicht – wie gewöhnlich – matt, sondern weist einen metallischen Glanz auf, sie irisiert.

Bei mikroskopischer Untersuchung mit Ölimmersion ist die Farbveränderung noch augenfälliger. Besonders an den dichteren Stellen, wo die E in Haufen liegen und wo Geldrollenbildung und Agglomeration am stärksten sind, tritt eine auffallende Verfärbung der E ein: anstelle von rosarötlich sind sie grau-grün bis grün. Es handelt sich dabei nicht um eine Polychromasie, wie sie bei anämischen Myelomkranken erkennbar ist, sondern um eine echte Metachromasie.

Auch an den Leukozyten werden häufig, wenn auch nicht konstant, Verfärbungen beobachtet: das Zytoplasma der Granulozyten, Lymphozyten und Monozyten erscheint beinahe farblos.

Die physikalischen Veränderungen sind nur an den Erythrozyten wahrnehmbar. Sie sind ebenfalls in Zonen stärkerer Anhäufung der E mit Geldrollenbildung und Agglomeration zu beobachten (Abb. 1). Hier sind die E wie von einem wolkigen Hauch überzogen, fliessen zusammen und sehen wie synzytiale Massen aus. Die sphärischen

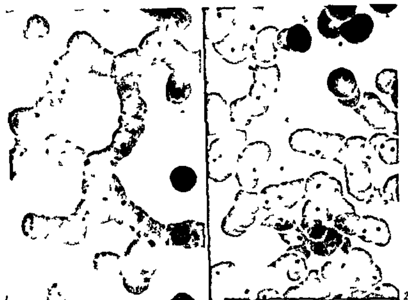


Abb 1 Plasmozytom mit Paraproteinämie Blutausstrich Giemsa Färbung Feste Geldrollenagglutinate im Original grünlich verfärbter miteinander verbackener unscharf begrenzter Erythrozyten × 1250
Abb 2 Dysproteinämie bei einem Patienten mit Leberzirrhose Blutausstrich Giemsa Färbung Geldrollenbildung normal scharf umrissener im Originalpräparat rosa gefärbter Erythrozyten (× 1250

Agglomerate machen den Eindruck von Öltropfen auf Wasser Bei Dysproteinamien die gleichfalls zu Geldrollenbildung und Agglomeration fuhren bleiben hingegen die scharfen Umrisse der einzelnen E gut erhalten (Abb 2)

Die beschriebenen Veranderungen habe ich zunachst bei bekannten Plasmozytomfallen gefunden so dass ich sie als fur die Krankheit pathognomonisch ansah Dann bin ich dazu ubergegangen systematisch bei noch nicht diagnostizierten Fallen nach diesen Veränderungen zu suchen und im Verdachtsfall die Spezialuntersuchungen durchzufuhren wodurch bis dahin unbekannte Plasmozytome gefunden oder Fehldiagnosen berichtigt werden konnten Der erste derartige Fall sei kurz angeführt

Am 3 10 1963 durchmusterte ich Blutausstriche verschiedener Patienten der Mediz Klinik des Vasile-Roaita Krankenhauses Sämtliche Ausstriche waren vom selben Tag und wurden gleichzeitig mit der gleichen Technik fixiert und gefärbt Ein Ausstrich fiel durch die geschilderten Veränderungen auf und liess an die Möglichkeit eines Plasmozytomes denken Die Aufnahmediagnose lautete Hypertonie, Knochen und Gelenkrheumatismus

Die Sternalpunktion bestätigte die Diagnose Plasmozytom Über 70% der kernhaltigen Markzellen waren typische Plasmozyten Es bestanden ferner eine Hyperproteinämie von über 9 g% und eine Hypergammaglobulinämie von 60% mit typischer Myelomzacke Die Röntgenaufnahmen zeigten eindeutige Knochenveränderungen und die Immunelektrophorese ergab ein Gamma Myelom

Diese Veränderungen, die ich anfangs nur bei Myelomfällen beobachtete, fand ich in der Folge auch bei anderen Paraproteinämien, so bei Waldenstromscher Krankheit und bei Begleitparaproteinämien anderer Erkrankungen (Leukämien) Sie kommen aber nur bei Paraproteinämien und nicht bei Dysproteinämien vor

Diskussion

Die beschriebenen Veränderungen sind m E durch Paraproteine bestimmter Art bedingt, welche die Blutkorperchen umhüllen Der auf diese Weise entstandene Film von Paraproteinen verändert einerseits die färberische Affinität der Erythrozyten und Leukozyten, führt zur beschriebenen Metachromasie und erzeugt andererseits die physikalischen Veränderungen der Erythrozyten

Es handelt sich offenbar um besondere Paraproteine, denn das Phänomen war nicht zu beobachten 1 bei Fällen von akuter und chronischer Entzündung, Tuberkulose, chronischen Hepatitiden, Leberzirrhosen, Kollagenosen, Hämopathien usw , die mit hochgradiger Dysproteinämie einhergingen, 2 bei lymphatischer Leukämie mit sicherer pathologischer Begleitmakroglobulinämie, 3 bei *Ago-globulinämie* mit rascher Gelifizierung des Blutserums bei Zimmertemperatur, mit starker Geldrollenbildung und E-Verklumpung, 4 auch bei vereinzelten sicheren Plasmozytomen

Es bleibt noch festzustellen, ob die beschriebenen Veränderungen vor anderen Symptomen der Krankheit erscheinen, oder aber als Ausdruck der bereits bestehenden Paraproteinämie zu deuten sind ob sie also eine Frühdiagnose oder aber, bei bereits entwickelter Krankheit, eine Schnelldiagnose ermoglichen

Summary

The author describes changes which have not previously been referred to in the red corpuscles in simple smears stained with the Giemsa method, these changes occur primarily in plasmocytoma, but may also be observed in other forms of paraproteinaemia, and may be of diagnostic value The question as to which abnormal protein fractions are responsible for the phenomenon is not yet settled

Zusammenfassung

Es werden bisher nic iebene Veränderung der roten Blutkorperchen im ein-
fachen, nach Giemsa get utausstrich besprochen, die in erster Linie bei Plasmo-
zytomen, aber auch bei Paraproteinämien vorkommen und von diagnostischer
Bedeutung sein können I welche anomalen Proteinfraktionen das Phänomen
erzeugen, ist noch nicht g

Adresse des Autors Dozent Dr a (Rumänien)

Current Problems in Immunology: O. WESTPHAL, H.-E. BOCK, E. GRUNDMANN (ed.) Springer, Berlin/Heidelberg/New York 1969, XI+349 pp., 135 fig.; DM 48.-/US $ 13.20

The proceedings of this 1st Bayer-Symposium under the heading 'Current Problems in Immunology' contain 33 papers. The book has 2 parts, a theoretical and a clinical one, and is centered, at least after the opening remarks, on immune diseases. The volume bears witness to the quality of immunological research in West Germany today as about two thirds of the authors come from that country. It also gives a cross section of some of the immunological research going on there. Most of the papers contain original results, practically all are stimulating to read and on a high level of quality. The papers are well presented and illustrated, however, the lack of summaries in about two thirds of them is an unpleasant omission. The main problem with this book is a general one and concerns proceedings of meetings without really restricted topics. To have the excellent paper on 'structure and formation of antibodies' by HILSCHMANN *et al.* with detailed information on aminoacid sequences of the variable parts of the L-chains, one has to buy at the same time papers about 'qualitative serum globulin determinations in chronic hepatitis' and on 'lymphocyte transformation of premature and mature infants'. To read the beautiful paper on 'Immunogenicity of semisynthetic penicillins' by WELLENSIEK means to get in addition the excellent report on 'Anticomplementary activity of guinea pig serum euglobulin' by KLEIN which perhaps is irrelevant for the reader concerned. The exciting atmosphere of a meeting, where basic immunologists of different fields and clinicians with their main interest in immunology come together, is not often found reading the papers afterwards: the variety of topics becomes a handicap. It is an enjoyable and stimulating book to read if one has the time, but it is not useful as a reference source nor if one wants to know where we stand with complement, lymphocyte transformation, drug allergies, immune diseases or any of the other topics, with a few exceptions. T. L. VISCHER, *Basel*

T. K. WITH: **Bile Pigments.** Chemical, Biological and Clinical Aspects. Academic Press, New York/London 1968. $ 12.00/112 s.

Das vorliegende Werk ist eine überarbeitete und erweiterte Ausgabe der im Thieme Verlag 1960 erschienenen «*Biologie der Gallenfarbstoffe*» in englischer Sprache. Wie der Titel sagt, werden chemische, biologische und klinische Aspekte des Gallenpigmentstoffwechsels besprochen. Der Stoff ist in folgende Kapitel gegliedert: Chemie, Bildung und Stoffwechsel im Körper, quantitative Daten der Gallenpigmentbildung, Ikterus, wobei hier auch die verschiedenen klinischen Ikterusformen besprochen werden. Es folgt ein ausführliches Kapitel über die Gallenpigmente im Blut, in welchem u. a. das Wesen der Diazoreaktion, der Konjugation, die normalen Serumbilirubinwerte, die angeborenen Störungen (hereditäre, nicht hämolytische Hyperbilirubinämien) und die Therapie behandelt werden. Die nächsten 4 Kapitel sind den Gallenpigmenten im Urin, in der Galle, im Stuhl und in Gewebsflüssigkeiten, Sekreten und Organen gewidmet. Hier wird auch der Kernikterus besprochen. Ein interessantes weiteres Kapitel ist der vergleichenden Biologie der Gallenpigmente bei Wirbeltieren, niederen Tieren und Pflanzen gewidmet. Eine Zusammenfassung über den klinischen Wert der verschiedenen Gallenpigmentbestimmungen sowie eine ausgedehnte Bibliographie von über 80 Seiten beschließen die Abhandlung zusammen mit einem ausführlichen Autoren- und Inhaltsverzeichnis.

Man kann den Autor zu dieser enzyklopädischen Behandlung des komplizierten und sich auf viele Gebiete erstreckenden Stoffs nur beglückwünschen. Ein Vergleich mit den

früheren Monographien desselben Autors über das gleiche Thema macht die großen Fortschritte, die auf diesem Gebiet in den letzten 10 Jahren mit der Anwendung neuer Methoden, vor allem der radioaktiven Gallenpigmentisotopen und neuartiger Analyseverfahren, erreicht wurden, sehr deutlich. Die kurze Aufführung der Kapitelüberschriften kann natürlich nur einen ganz oberflächlichen Einblick in die behandelten Gebiete geben. Es ist wohl nicht übertrieben zu sagen, dass kaum eine Frage der Physiologie oder Klinik der Gallenfarbstoffe unbeantwortet bleibt oder, sofern sie noch nicht gelöst ist, wenigstens diskutiert wird. Demgegenüber ist die Chemie der Gallenfarbstoffe relativ kurz, aber für das Verständnis der nachfolgenden Ausführungen genügend abgehandelt. Ausserdem erlaubt die ausführliche Angabe des verwendeten Schrifttums, das den Grossteil der in den verschiedenen Sprachen veröffentlichten Arbeiten umfasst, jederzeit die Originalarbeiten zu konsultieren. Wer sich mit den Gallenpigmenten und dem Ikterus entweder in der Klinik oder in der Forschung befasst, wird wohl dieses Werk immer wieder mit Gewinn zu Rate ziehen.

M. VEST, *Basel*

Varia

Hungarian Haematological Society

The 5th Congress of the Hungarian Haematological Society will be held in Budapest May 26–28, 1971 Apart from plenary sessions where invited speakers will deliver special lectures in English, there will be English speaking symposia on platelet kinetics and physiopathology, immunologically functioning cells, and structure and function of red cells *President of the Congress* E. Keleman, M D , D Sc

Colleagues who are intended to deliver a lecture and/or to participate, or to have further information are asked to write to the address Haematological Congress, MOTESZ, *Apród u 1–3, Budapest I (Hungary)*

International Society of Haematology
Asian-Pacific Division

The Second Meeting of the Asian Pacific Division will be held at the University of [illegible]

blood transfusion and immunohaematology A number of special sessions are planned on a wide range of subjects

The president, Dr John H Colebatch and the organizing committee extend a cordial invitation to all haematologists to visit Melbourne for the meeting Details may be obtained from the Haematology Secretariat, P O Box 29, *Parkville, Victoria, 3052* (Australia)

Acta haemat. 44: 193–199 (1970)

Studies in Iron Absorption[1]

VII. Iron Deficiency in Young Men

S. Höglund, L. Ehn and Gudrun Liedén

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It is well known that iron deficiency is a common condition especially in young women, while men are usually considered not to develop iron deficiency under normal conditions [1]. The daily dietary intake of iron is supposed to be sufficient to cover the daily iron losses (0.5–1 mg) in men [2]. However, the rapid growth during adolescence may increase iron requirements, and it has been claimed that a negative iron balance might occur even in men [3, 4].

The aim of the present investigation was to study the iron situation in young men. The following parameters of iron metabolism were studied: hemoglobin concentration, packed red cell volume, reticular bone marrow iron (hemosiderin) and sideroblast count, serum iron concentration, total iron binding capacity (TIBC) and iron absorption.

Material

Present group. 44 men aged 18 to 26 years were studied. They were all newly-drafted conscripts at the same infantry regiment. All of them were apparently healthy and had passed a general physical examination without remarks: none was a blood donor, none had suffered from gastric ulcer or any other severe disease. About half of the subjects came to military service directly from school or had been occupied with clerical jobs. The rest of the subjects had been working at different kinds of manual labor, only few had been heavy workers. About $^{1}/_{3}$ of the subjects admitted participating in active sport.

Controls. To establish normal values of hemoglobin, serum iron and TIBC 91 healthy male volunteers were used. The normal values of iron absorption were obtained in 24 healthy men: the material and method were described in detail earlier [5].

[1] Supported by Astra, Södertälje.

Methods

Hemoglobin concentration and packed red cell volume were measured by standard methods previously described [6] The hemoglobins of the controls were analyzed at another laboratory using the same method Checked against the international ICSH standard the two laboratory routines gave identical results Serum iron and TIBC were analyzed on a Technicon autoanalyzer with tripyridyl triazine as colour-developing reagent according to the methods described by YOUNG and HICKS [modification BABSON–KLEINMAN] and HENRY *et al* [7, 8, 9] Iron stores were studied by estimating the stainable iron and by counting the sideroblasts in bone marrow smears according to the method described by HANSEN and WEINFELD [10] For the stainable iron a scale from 0–4 was used Sideroblasts were registered as more or less than 12% [11]

Iron absorption was studied by means of radioactive iron and a whole body counter The method and normal values have been described in detail [5] Briefly a test dose of 0.25 mg ferrous iron in the form of ferrous sulphate labelled with 0.5–1 μCi ^{59}Fe is administered orally and the body retention of the test dose is measured in the whole body counter 10–14 days after administration The absorption in percent of the test dose can than readily be deduced

Results

Peripheral blood values The mean peripheral blood values are shown in table I The hemoglobin of the present group is significantly lower (p < 0 001) than that of the older controls but also lower than that of the part of the controls of similar age Serum iron and TIBC values are not distinguished from the controls

Hemosiderin, sideroblasts and absorption The stainable iron in bone marrow smears is shown in figure 1 Seven of 44 subjects showed lack of stainable iron or only traces of iron The mean score for the whole group was 2 3 The sideroblast count was more than 12% in 40 of the subjects and less than 12% in 4 of the subjects Table II

Table I Iron situation in young men Peripheral hematologic values (M ± SE)

	No	Age, years range and mean	Hemoglobin g/100 ml	Packed cell volume %	Serum iron mg/100 ml	TIBC, mg/100 ml
Present group	44	18–26 21 7	13 9 ± 0 12	42.8 ± 0 34	0 103 ± 0 005	0 355 ± 0 007
Controls same age	42	18–26 21 8	15 0 ± 0 11	–	0 111 ± 0 004	0 352 ± 0 009
Controls older	91	18–46 30 3	14.8 ± 0 09	–	0 110 ± 0 004	0 368 ± 0 00[illegible]

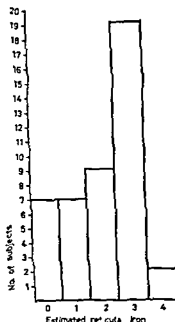


Fig. 1 Iron situation in young men. Reticular bone marrow iron.

Table II Iron situation in young men: absorption of iron

Subject category	No.	Age years (mean)	Iron absorption % of 0.25 mg Fe++ M ± SE	Significance of difference between means compared with controls
Present group	44	21.7	31.7±2.2	p<0.001
Controls	24	28.6	19.0±2.3	–
Blood donors (mean 40.5 donations)	41	33.4	48.1±4.3	p<0.001

shows that in the present group of subjects with an average age of 22 years iron absorption is significantly higher than among controls aged 29 years. For comparison the absorption figures of a group of blood donors are shown which are still higher [12].

Correlation between iron absorption and other parameters studied. It is shown in table III that iron absorption in the present group is sig-

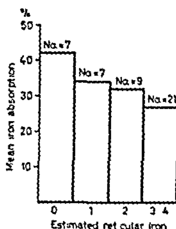


Fig. 2. Iron situation in young men. Correlation between iron absorption and storage iron.

nificantly correlated to TIBC and to the iron stores, as they are reflected by bone marrow iron, but not to the other factors. The close relationship between iron stores and iron absorption is further illustrated in figure 2. It shows that the subjects exhibiting trace or less of reticular bone marrow iron have higher iron absorption than the whole group. In fact, these healthy young men absorb as much iron as blood donors with exhausted iron stores (table II) [12].

Discussion

The significantly lower hemoglobin values in the present group compared with the controls suggest that a tendency to anemia may exist although only one of the subjects had a hemoglobin value below 12.6 g/100 ml. Unfortunately control values of packed red cell volume were not available. It has, however, been shown [6] that hemoglobin and packed red cell volume follow each other rather closely and it is probable that the figure obtained in the present group, 42.8%, is somewhat low.

Both the present group and the controls showed wide ranges in serum iron and TIBC values, the SD of serum iron being 0.039 mg/100 ml and 0.034 mg/100 ml, respectively. On the 95% confidence level, the controls would have serum iron between 0.042 mg/100 ml and 0.178 mg/100 ml. All serum iron values of the subjects in the present group of younger men appeared within these limits. The wide

Table III Iron situation in young men. Correlation between iron absorption and other parameters of iron metabolism

Parameter	Correlation coefficient	Statistical significance of correlation
Hemoglobin	−0 16	p>0 05
Packed cell volume	+0 16	p>0 05
Serum iron	+0 08	p>0 05
TIBC	+0.30	0 05>p>0 01
Reticular bone marrow iron	−0.32	0 05>p>0 01

dispersion of serum iron and TIBC values in different groups of subjects have been discussed earlier [5] It is obvious that isolated determinations of serum iron are of limited value in the diagnosis of iron deficiency The present findings support this view

Lack of stainable reticulo endothelial iron in bone marrow smears is often considered to be synonymous with iron deficiency [1, 11] 16% of the subjects of the present group showed absence or traces of stainable iron This does not necessarily prove that their iron stores are exhausted, but indicates decreased stores or iron deficiency Also the sideroblast count seems to indicate depletion of iron stores in this group of subjects Only small stores of iron are supposed to be needed to provide a normal sideroblast count [11] but lower values are found in 10% of the cases However, out of the 4 cases with low sideroblast counts, 2 had simultaneously normal amounts of reticular iron and normal iron absorption A low sideroblast count thus does not necessarily indicate sideropenia

It has been shown that the iron absorption test is a sensitive criterion of early iron deficiency [5, 12] Healthy fertile women and non anaemic and otherwise healthy blood donors exhibit increased iron absorption compared to male controls [5, 12] This has been regarded as an indication of increased iron requirements among subjects of these categories

The young men investigated here thus have lower hemoglobin than the controls but no anaemia and they do not differ from controls concerning serum iron and TIBC values However, the degree of stainable reticulo-endothelial bone marrow iron sideroblast count

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Hydroxyproline Excretion in Iron Deficiency Anaemia prior to and after Substitution with Iron-Dextran[1]

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Iron deficiency clearly affects other haemoproteins than haemoglobin [5]. Thus, low tissue concentrations of cytochromes, catalase, and myoglobin have been reported. Moreover, iron is not only a structural component of organic molecules, but also a cofactor in enzymatic reactions. For example, iron dependency of glutamate-forminino-transferase has been related to megaloblastic maturation in iron deficiency anaemia [14]. Furthermore, activity of the protocollagen-proline-hydroxylase requires the presence of ferrous iron [3, 12]. It appeared justified, therefore, to investigate a selected aspect of collagen metabolism in iron deficiency anaemia. In this paper data on hydroxyproline excretion prior to, and following correction of iron deficiency with a single large dose of iron-dextran are reported.

Patients and Methods

Ten patients suffering from iron deficiency anaemia and 5 haematologically normal controls were studied (tables I and II). Iron deficiency was established by the following criteria: hypochromic microcytic anaemia, low iron and high transferrin levels, absence of bone marrow iron, and adequate response to iron treatment. Patients and controls were adults. Control subjects had normal haemoglobin concentrations and normocytic blood smears. In the 2 alcoholics biopsies revealed normal liver histology.

Patients and controls were hospitalized and kept on hydroxyproline free diet during 6 days. Meat, fish, gelatine-containing foods and sweets were specifically excluded. 24 h samples of urine were collected at day 3 and 6. On day 4 iron-dextran was administered intravenously in 500 ml of saline over a 2 h period. Controls received 1 g of iron, iron deficient subjects according to the severity of anaemia, 1.5 to 3 g [2].

[1] Supported in part by Schweizerischer Nationalfonds zur Förderung der wissenschaftlichen Forschung.

Table I Clinical and haematological details of patients

Case No.	Sex	Age years	Haemoglobin g per 100 ml	Serum iron µg per 100 ml	Total iron binding capacity µg per 100 ml	Marrow iron	Cause of iron deficiency
1	F	32	5.3	14	3[illegible]8	absent	Menorrhagia
2	F	41	8 7	15	360	absent	Menorrhagia
3	F	42	9.5	37	477	absent	Duodenal ulcer
4	F	70	8 9	21	5[illegible]8	absent	Hiatus hernia
5	M	49	7 7	13	490	absent	Colitis
6	M	63	9 4	20	435	absent	Unknown
7	M	65	9 0	12	336	absent	Colon diverticulosis
8	M	77	5 2	20	420	absent	Partial gastrectomy
9	M	77	8 1	24	438	absent	Hiatus hernia
10	M	87	7 1	9	384	absent	Gastric ulcer

Table II Clinical and haematological details of control subjects

Case No.	Sex	Age years	Haemoglobin g per 100 ml	Clinical diagnosis
1	F	58	13 7	Essential hypertension
2	M	23	17 0	Atrial septum defect
3	M	33	17 6	Functional dysphagia
4	M	35	15.2	Alcoholism
5	M	38	16 9	Alcoholism

Urine hydroxyproline was quantitated accord[illegible]
modification After [illegible]
to an ion exchanger [illegible]
into pyrex test tube [illegible]
1×8 200/400 Mesh [illegible]
Plasma iron and tota[illegible]
[10] Haematologic[illegible]
statistical analysis th[illegible] was employed

Results

Mean daily urinary hydroxyproline prior to iron-dextran was 28 7 mg for patients, and 25 6 mg for controls (table III) This dif-

Table III Daily urinary hydroxyproline excretion prior to and following iron-dextran in patients and controls

Case No	Hydroxyproline excretion mg per 24 h	
	Prior to Fe-dextran	*After Fe-dextran*
Patients		
1	23.8	32.4
2	30.4	37.8
3	29.0	54.8
4	22.6	29.9
5	21.6	32.1
6	38.4	44.8
7	33.8	51.5
8	31.9	36.5
9	36.9	48.0
10	18.8	26.9
Mean	*28.7*	*39.4*
Controls		
1	19.9	20.2
2	30.1	29.7
3	27.6	27.4
4	30.0	30.8
5	20.5	20.4
Mean	*25.6*	*25.7*

ference was statistically not significant ($P > 0.15$). While on the second day after 1 g of iron hydroxyproline excretion was unchanged in control subjects ($P > 0.3$), there was a significant increase to an average value of 39.4 mg in all patients in response to correction of iron deficiency ($P < 0.0025$).

Discussion

The presence of hydroxyproline is essentially restricted to collagen. Urine hydroxyproline is predominantely in peptide form [18], and, under hydroxyproline free diet, quantitatively reflects collagen breakdown [15], although large amounts of catabolites are excreted as respiratory carbon dioxide. Collagen synthesis first involves formation of the proline and lysine containing protocollagen on the polysomal level, and, after chain completion, and release, hydroxylation of both amino acids [13]. Activity of protocollagen-proline-hydroxylase requires the presence of oxygen, ascorbic acid, and ferrous iron [3, 12,

13] If connective tissue cells are incubated in presence of chelators of ferrous iron protocollagen accumulates intracellularely [3, 12] Protocollagen apparently cannot pass out of the cell until the hydroxyl groups are added [7] In the adult organism collagen formation, and breakdown are in equilibrium, but different collagen pools with differing turnover rates exist [13] Thus, synthesis of young tropocollagen is accompanied by significant simultaneous degradation [4, 8, 12], relatively great amounts of these catabolites appearing in the urine [12, 13] Upon establishment of the tertiary structure the molecule becomes more stable, turnover is low, and breakdown products largely take the respiratory route [11, 17]

In 10 severely iron deficient individuals daily urinary hydroxyproline averaged 28 7 mg, compared to 25 6 mg in 5 controls, being well within the physiological range of 15 to 55 mg [6, 9] *In iron* deficiency, 2 days after parenteral administration of 1 5 to 3 0 g of iron, hydroxyproline excretion increased by almost 40% No change occured in the control subjects in response to 1 g of iron, pointing to the causal relationship between the correction of the deficiency state, and the changing pattern of hydroxyproline excretion Induction of a negative collagen balance cannot be excluded as causal for this phenomenon It is tempting, however, to postulate another mechanism restauration of an adequate iron supply to connective tissue cells leads to rapid conversion of the accumulated protocollagen into collagen, a sequence observed when isolated tissue incubated without oxygen is exposed to air [7], despite the rapid breakdown of some of the hydroxylated molecules [4, 8], a positive collagen balance would result Normal base line excretion of hydroxyproline *in iron deficiency* may be explained by the fact, that large changes in collagen catabolism are required to alter the rate of hydroxyproline excretion [11, 17]

At the present time no clinical condition associated with iron deficiency can be related to a disturbed collagen metabolism Animal experiments showed a defect *in* collagen formation in skin wounds, if *iron* deficiency was present [1] It seems justified to be aware of related disturbances in human pathology

Summary

Base line urine hydroxyproline was found in the physiological range in 10 patients with iron deficiency anaemia 2 days after infusion of iron-dextran it had increased by

almost 40%. No such response to iron-dextran was observed in 5 haematologically normal individuals. This finding is discussed in the light of the known dependency of protocollagen-proline-hydroxylase on iron.

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Lysosomale Enzyme in Lymphozyten

I. Lymphoretikuläre Erkrankungen: Vergleich des Enzymgehaltes (saure Phosphatase, β-Glucuronidase) unstimulierter Blutlymphozyten mit der Blastentransformation nach Phytohämagglutinin-Stimulierung *in vitro*[1]

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Blutlymphozyten gesunder Spender lassen nach Stimulierung mit Phytohämagglutinin (PHA) *in vitro* zahlreiche morphologische und metabolische Veränderungen erkennen, die bereits wenige Minuten bis Stunden nach Zusatz des Agens (Frühveränderungen) oder erst nach ein bis mehreren Tagen nachweisbar werden [27]. Während der Frühphase der Stimulierung zeigen die in Lymphozyten vorhandenen Lysosomen eine Labilisierung [21, 33]. Die Bedeutung dieser Lysosomenveränderungen ist bisher ungeklärt. Es wird diskutiert, dass durch die Labilisierung die Teilnahme lysosomaler Enzyme an Stoffwechselvorgängen ermöglicht und/oder erleichtert wird, die für die Umwandlung ruhender Lymphozyten in blastenartige Zellen mit gesteigerter Mitoseaktivität wesentlich sind [21, 22].

Blutlymphozyten von Patienten mit chronischer lymphatischer Leukämie (CLL) zeigen eine deutlich verminderte und verzögert einsetzende Stimulierung nach Zusatz von PHA [5, 13, 16, 35, 42a, 46]. Es ist weiterhin bekannt, dass Lymphozyten von Patienten mit anderen neoplastischen und benignen lymphoretikulären Erkrankungen, zum Beispiel Morbus Hodgkin [17, 18, 42, 44], Paraproteinämien (Plasmozytom, Makroglobulinämie Waldenström [12, 43]), infektiöser Mononukleose [41] und Morbus Boeck [19, 27], in ihrer *in vitro*-Reaktivität auf PHA von der Norm abweichen können.

[1] Herrn Prof. Dr. O. H. Arnold zum 60. Geburtstag in Dankbarkeit gewidmet.
Auszugsweise vorgetragen bei der 76. Tagung der Deutschen Gesellschaft für Innere Medizin (Wiesbaden, 6.–9. April 1970).

Eigene biochemische Untersuchungen [7] ergaben, dass die Aktivität der lysosomalen Hydrolasen saure Phosphatase und β-Glucuronidase in unstimulierten CLL-Lymphozyten durchschnittlich niedriger ist als in entsprechenden normalen Zellen. Diese Ergebnisse stimmen mit zytochemischen Befunden anderer Autoren überein [15, 28, 52]. Die Bedeutung älterer biochemischer Studien mit ähnlichen Resultaten wird dadurch etwas eingeschränkt, dass sie nicht an reinen Lymphozytenpräparationen vorgenommen wurden [1, 3, 14]. Die Aktivitätsminderung lysosomaler Hydrolasen lässt sich anderen Stoffwechseldefekten und funktionellen Störungen zuordnen, die bei CLL-Lymphozyten beschrieben wurden und diese Zellen als pathologisch charakterisieren [8, 9, 11, 24, 26, 32, 37, 38, 39, 45, 48, 49, 51].

Es ist bekannt, dass CLL-Lymphozyten von Patienten *mit hohen* Blutlymphozytenzahlen auf eine 3 tägige Inkubation mit PHA schwächer reagieren als Zellen von Patienten mit nur geringer Lymphozytose [4, 16, 35, 38, 42a, 46]. Darüber hinaus wurde festgestellt, dass bei CLL-Patienten eine durch zytostatische Therapie erzielte Reduktion der Blutlymphozytenzahl mit einer Zunahme der PHA-Stimulierbarkeit einhergehen kann [35]. Aus diesen Beobachtungen kann geschlossen werden, dass im peripheren Blut von CLL-Patienten normale Lymphozyten und Zellen mit verminderter PHA-Empfindlichkeit («pathologische» Zellen) vorkommen, deren Verhältnis sich mit steigender Gesamtlymphozytenzahl zugunsten der pathologischen Zellen verschiebt. Es ist zu vermuten, dass die bei CLL-Patienten beobachtete Verminderung der mittleren Aktivität der lysosomalen Enzyme saure Phosphatase und β-Glucuronidase dadurch zustande kommt, dass enzymarme Lymphozyten in grösserer Zahl als Zellen mit normaler Enzymausstattung vorhanden sind.

In den hier mitgeteilten Untersuchungen wurde geprüft, ob bei der CLL der Gehalt der Lymphozyten an saurer Phosphatase und *β-Glucuronidase* wie die *PHA-Stimulierbarkeit der Zellen* mit der Gesamtlymphozytenzahl negativ korreliert ist. Ausserdem interessierte die Frage, ob eine positive Korrelation zwischen der Enzymausstattung unstimulierter Lymphozyten und ihrer Reaktion auf PHA nach 3 tägiger Stimulierung besteht. Da eine derartige Korrelation nur dann auf einen kausalen Zusammenhang zwischen dem Enzymgehalt und der Blastentransformation hinweisen könnte, wenn sie nicht nur bei der CLL, sondern auch bei anderen Erkrankungen mit gestörter PHA-Reaktivität nachweisbar wäre, wurden in die Untersuchungen auch

Lymphozyten von Patienten mit Morbus Hodgkin, Paraproteinämie, Morbus Brill-Symmers, infektiöser Mononukleose und Morbus Boeck einbezogen.

Material und Methoden

Patienten Die Untersuchungen wurden an 3 gesunden Blutspendern und 18 Patienten mit lymphoretikulären Erkrankungen durchgeführt. Bei letzteren handelte es sich um

[illegible]

Anämie Prednison Bei keinem der Patienten mit Paraproteinämie waren Plasmazellen im peripheren Bl[illegible] nachweisbar [illegible]

[illegible]

1 256, nur bei dem zuerst genannten Patienten waren noch einzelne atypische mononukleäre Elemente im peripheren Blut zu beobachten. Der 3. Patient (M M) wurde 10 Tage nach Auftreten der klinischen Symptomatik untersucht. Im peripheren Blut fanden sich bei einer Gesamtleukozytenzahl von 11500/µl 45% atypische mononukleäre Zellen, heterophile Antikörper konnten zu diesem Zeitpunkt noch nicht nachgewiesen werden.

Zellgewinnung Die Zellgewinnung erfolgte nach einer früher beschriebenen Methode [6] und umfasste folgende Schritte Spontansedimentation der Erythrozyten in frisch entnommenem, heparinisierten Venenblut bei 37°C während 1-2 ½ Stunden Abpipet-

[illegible]

[illegible], Grand Island, N Y , USA) und Resuspendieren in komplettem [illegible]
(=MEM-S [illegible]
10000 IE Pe[illegible]
zunächst 12–[illegible]
schliessend in einer verbesserten Neubauer-Kammer durchgeführt, die Reinheit der Zellsuspensionen an Ausstrich-Präparaten (Pappenheim-Färbung) untersucht und die Vitalität der Lymphozyten im Farbstoffausschluss-Test mit Erythrosin B geprüft.

Kulturverfahren und präparative Massnahmen Die für die biochemischen Studien bestimmten Kulturen enthielten $0{,}91 \pm 0{,}12 \times 10^6$ Zellen/ml Kulturmedium (Gesamtvolumen 40 ml)

[illegible]

wurden den Kulturen 0,02 ml/ml Medium zugesetzt. Durch Vortestung der verwendeten PHA-P Charge war gezeigt worden, dass mit dieser PHA Konzentration eine optimale Blastentransformation erzielt werden konnte. Als Kontrollen dienten Lymphozytensuspensionen, die mit 0,02 ml einer 0,9%igen NaCl Lösung/ml Kulturmedium behandelt wurden.

Die für die biochemischen Untersuchungen vorgesehenen Zellsuspensionen wurden im Eisbad gekühlt, mit 15 ml MEM-S (4°C) versetzt und bei 724 $g \times 15$ min zentrifugiert. Die im Sediment noch vorhandenen Erythrozyten liessen sich durch einen 2- bis 3 maligen hypotonen Schock lysieren. Die Zellen wurden anschliessend in 3,7 ml einer kalten (4°C) Saccharose (0,34 M) - EDTA (0,01 M) - Lösung (pH 7,0) resuspendiert und durch 10 maliges Einfrieren (Kohlensäureschnee-Aceton Gemisch) und Auftauen homogenisiert. Die Freisetzung der in subzellulären Organellen enthaltenen Enzymaktivitäten war nach 15 minütiger Einwirkung von Triton X-100 (Endkonzentration 0,1%) erreicht. Die mit Triton X-100 behandelten Homogenate wurden bei 20000 $g \times 10$ min zentrifugiert und der resultierende Überstand für die biochemischen Analysen verwendet.

Zur Beurteilung der Blastentransformation wurden die Kulturen nach der von Hirschhorn [20] angegebenen Methode (ohne vorherigen Zusatz von Vincaleukoblastin) mit einem Alkohol-Essigsäure-Gemisch fixiert und mit saurem Orcein (0,5%) gefärbt. Die Auswertung fand im Phasenkontrastmikroskop statt. Als zusätzliche Messgrösse für die durch PHA induzierte Stimulierung wurde in einem Teil der Kulturen der Einbau von ^{3}H-Thymidin (^{3}H TdR) in die Lymphozyten bestimmt [Methode siehe 25, 34].

β-Glucuronidase. Die Bestimmung erfolgte bei pH 4,6 nach einer Modifikation der von Talalay et al. [47] beschriebenen Methode. Als Substrat diente Phenolphthalein Glucuronsäure (0,01 M, Sigma Chem. Co., St. Louis, Mo. USA), die Inkubationszeit betrug 6 Stunden [Einzelheiten siehe 6].

Saure Phosphatase. Die Bestimmung wurde bei pH 5,0 nach einer Modifikation der von Valentine und Beck [50] beschriebenen Methode vorgenommen. Als Substrat diente D, L-β-Glycerophosphat (0,052 M, Sigma Chem. Co.), die Inkubationszeit betrug 6 Stunden. Das freigesetzte anorganische Phosphat (Pi) wurde nach einer Modifikation der Methode von Chen et al. [10] bestimmt [Einzelheiten siehe 6].

Malat Dehydrogenase. Die Bestimmung erfolgte nach der von Meisler et al. [31] beschriebenen Methode. Anstelle des in der Originalmethode angegebenen Glycyl Glycin Puffers wurde Phosphat Puffer (0,25 M, pH 7,4) verwendet [Einzelheiten siehe 6].

Protein. Die Bestimmung erfolgte nach der Methode von Lowry et al. [29]. Als Standard diente gereinigtes Human Serumalbumin (Behring Werke, Marburg/Lahn). Nach Zugabe des Folin Ciocalteu Reagens bildete sich ein feiner, gelber Niederschlag, der auf die Anwesenheit von Triton X 100 in den Proben zurückzuführen war und durch Zentrifugation bei 20000 $g \times 10$ min entfernt werden konnte, ohne dass sich im Überstand die Extinktion bei 660 nm änderte.

Berechnungen. Für die Berechnung der Signifikanz von Unterschieden wurde der t-Test nach Student verwendet. Es werden stets der Mittelwert ($\bar{x}$) und die Standardabweichung des Mittelwertes ($s_{\bar{x}}$) angegeben. Bei den Korrelationsberechnungen wurde für die Signifikanz gegen Null eine Irrtumswahrscheinlichkeit von 5% zugrunde gelegt.

Ergebnisse

In den gereinigten Lymphozytensuspensionen von Patienten mit CLL fanden sich 100%, bei den übrigen Patienten und den Normalpersonen 98–99% mononukleäre Zellen. Den Lymphozytenpräpara-

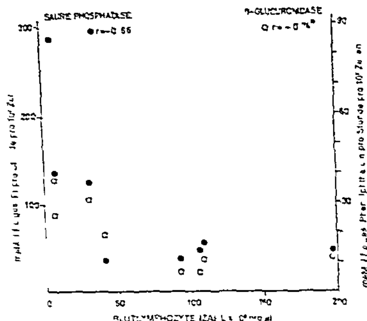


Abb. 1. Beziehung zwischen der Aktivität der sauren Phosphatase und β-Glucuronidase in unstimulierten Lymphozyten und der Blutlymphozytenzahl bei Patienten mit CLL. r = Korrelationskoeffizient. * = signifikant gegen Null.

tionen von Patienten mit infektiöser Mononukleose waren grössere atypische mononukleäre Formen nicht beigemischt, bei Morbus Hodgkin bestand gelegentlich eine Verunreinigung mit 1–2% eosinophilen Granulozyten. Zu Versuchsbeginn wurden in allen Lymphozytensuspensionen 97–100% vitale Zellen nachgewiesen.

In Abbildung 1 sind die in unstimulierten CLL-Lymphozyten nachgewiesenen Aktivitäten der sauren Phosphatase und β-Glucuronidase gegen die zum Zeitpunkt der Untersuchung im peripheren Blut der Patienten bestimmten Lymphozytenzahlen aufgetragen. Es zeigt sich, dass die Korrelationskoeffizienten für beide Enzyme relativ hoch und negativ sind, die Korrelation konnte jedoch nur für die β-Glucuronidase statistisch gesichert werden.

Tabelle I und Abbildung 2 geben den Proteingehalt und die Enzymaktivitäten (saure Phosphatase, β-Glucuronidase, MDH) wieder,

Tabelle 1 Aktivität von saurer Phosphatase, β-Glucuronidase und Malat Dehydrogenase (MDH) sowie [illegible] nach 3-, 5- und 7-tägiger Blastentransformation nach [illegible] Erkrankungen

Diagnose Name Alter/Geschlecht	Lymphozyten pro µl Blut	Therapie	Saure Phosphatase pro 10^7 Zellen	β-Glucuronidase pro 10^7 Zellen	MDH pro 10^7 Zellen
Normalpersonen (n = 3)	–	–	$\bar{x} \pm s_{\bar{x}}$ 153,28 ± 11,60	50,59 ± 6,9[illegible]	303,64 ± 43,[illegible]
CLL					
K.J., 63J., ♀	5 637	c	28[illegible],12	25,71	403,41
W.E., 66J., ♀	8 000	c	135,26	40,15	410,30
L.M., 67J., ♂	31 600	c	122,75	31,34	435,84
W.K., 56J., ♀	41 360	c	19,91	19,37	368,01
K.E., 54J., ♂	93 600	c	37,97	6,34	195,78
Sch.H., 70J., ♂	105 232	+	47,46	6,20	150,53
R.P., 47J., ♂	108 750	+	54,67	10,65	90,95
St.R., 54J., ♂	197 160	c	28,48 (19,91–28,48: P < 0,005)	12,14	183,93
			$\bar{x} \pm s_{\bar{x}}$ 91,70 ± 31,74 n.s.	18,99 ± 4,40 P < 0,005	280,48 ± 48,57 n.s.
Morbus Hodgkin					
W.H., 22J., ♂ Stad. II	637	c	103,41	30,47	114,13
K.J., 22J., ♂ Stad. II	1 853	c	68,82	3[illegible]	211,01
Sch.F., 21J., ♂ Stad. III	437	+	1[illegible]6,30	[illegible]6,35	194,93
W.H.J., 1[illegible]J., ♂ Stad. III	1 210	c	128,14	32,75	350,[illegible]
M.H., 30J., ♀ Stad. IV	1 116	c	171,08	32,12	1[illegible],57
			$\bar{x} \pm s_{\bar{x}}$ 1[illegible]9,56 ± [illegible]0,36 n.s.	30,89 ± 1,21 n.s.	199,52 ± 4[illegible],25 n.s.
Plasmozytom (IgG)					
G.J., 54J., ♂	1 148	c	130,51	48,60	20[illegible],56
R.B., 49J., ♂	1 440	+	139,05	41,58	1[illegible]2,40
W.H., 69J., ♂	853	–	116,57	43,56	3[illegible]0,19
Makroglobulinämie Waldenström (IgM)					
E.A., [illegible]6J., ♀	1 013	–	1[illegible]6,1[illegible]	42,16	2[illegible]9,18
St.T., [illegible]J., ♂	2 150	–	1[illegible]8,[illegible]1	34,34	241,29
			$\bar{x} \pm s_{\bar{x}}$ 148,20 ± 12,4[illegible] n.s.	42,05 ± [illegible],29 n.s.	2[illegible]0,1[illegible] ± 3[illegible],6 n.s.
Infektiöse Mononukleose					
B.H.J., 29J., ♂	2 370	c	[illegible]	18,67	25[illegible],05
S.M., 16J., ♀	2 208	c	109,94	15,14	1[illegible]7,[illegible]0
M.M., 19J., ♂	3 155	–	1[illegible]6	2,33	50,50
			$\bar{x} \pm s_{\bar{x}}$ 1[illegible]7,45 ± 22,65 n.s.	12,05 ± 4,9[illegible] P < 0,005	160,08 ± [illegible] P < 0,05
Morbus Brill-Symmers					
N.G., 50J., ♂	910	c	23[illegible],29	30,80	291,[illegible]0
Morbus Boeck					
Ch.J., 43J., ♂	1 512	c	2[illegible],12	58,46	435,20

1 Übereinstimmung mit ^3H Thymidin-Einbau

Proteingehalt in unstimulierten Lymphozyten von Gesunden und Patienten mit lymphoretikulären Er
Inkubation mit PHA und 0 9% NaCl Lösung (Kontrollen)

Protein pro 10⁷ Zellen	Transformationsrate (%) nach PHA Zusatz					
	3 Tage		5 Tage		7 Tage	
	PHA (NaCl)	Mitosen ‰	PHA (NaCl)	Mitosen ‰	PHA (NaCl)	Mitosen ‰
139 43 ± 14 62	53 6 ± 8 6 (0,8 ± 0 6)	4 6 ± 2 3	58 1 ± 15 4 (1 4 ± 0 7)	7,3 ± 1,3	47 4 ± 15 6 (0 6 ± 0,8)	7 0 ± 1,5
634,53	79,2 (0,2)	8 0	–	–	–	–
368,24	6 0 (1 0)[1]	0,0	73 8 (3,2)[1]	14 0	63 0 (0 4)[1]	2,0
233,56	0 6 (0 0)[1]	0 0	29,2 (0 0)[1]	0 0	36,2 (0 0)[1]	7 0
134 47	0,5 (0 0)	0 0	14 3 (0 0)	2,0	34 4 (0 9)	2 0
86,52	1 4 (0 0)[1]	0 0	15 4 (0,2)[1]	4 0	0,8 (0 0)[1]	0 0
132 85	4 4 (0,2)[1]	0 0	4,2 (0 0)[1]	0 0	4,8 (0 0)[1]	0 0
87 71	2 0 (0 0)[1]	0 0	16 0 (0 0)[1]	0 0	2 8 (0 0)[1]	0 0
54 67	1,8 (0 0)[1]	0 0	3 6 (0 0)[1]	0,0	3,2 (0 0)[1]	0,0
217 19 ± 69,65 n.s.	11,9 ± 9,5 (0,2 ± 0 1)	1 0 ± 1 0	22 4 ± 9,2 (0 5 ± 0,5)	2 9 ± 1 9	20 7 ± 9 1 (0,2 ± 0 1)	1 6 ± 1 0
120 14	70,9 (0 0)[1]	12,3	–	–	–	–
85 12	74 0 (0 1)	1 0	–	–	–	–
223,57	9 0 (0 0)	0 0	–	–	–	–
144 14	7,6 (0 0)	6 0	83 4 (2 6)	6 0	–	–
125 05	19 8 (0 0)[1]	2 0	–	–	–	–
139,59 ± 23 05 n.s.	35 1 ± 15,5 (0 0 ± 0 0)	5,3 ± 2 6				
250 17	27 4 (2 0	10 0	81,8 (5 6)	2 0	–	–
171 43	89 0 (0 0)	4 0	90 4 (1 6)	16 0	–	–
209 65	76 0 (0 3)	1 0	–	–	–	–
194 42	58,5 (2 0)	10 0	–	–	–	–
257,34	77,8 (0 0)[1]	0 0	–	–	–	–
216 60 ± 16,38 $P<0.01$	64 7 ± 11 7 (0 9 ± 0,5)	5 0 ± 2 1				
70,21	0,2 (0 0)[1]	0 0	0 6 (0 4)[1]	0 0	0 0 (0 0)[1]	0 0
87,82	63 0 (0 6)	1 0 (2 Tag)	–	–	–	–
53,56	7,9 (0 0)[1]	0 0	6 8 (0 0)[1]	0 0	7,2 (0 0)[1]	0 0
70,53 ± 9 89 $P<0.01$	23 7 ± 19 8 (0,2 ± 0,2)	0,3 ± 0 3				
327 97	34,8 (1 8)	8 0	87 0 (0,2)	6 0	–	–
294,57	26,8 (2,0)	16 0	94 0 (3,2)	3? 0	–	–

Aktivitätseinheiten Saure Phosphatase mµMol freies Pi/Std. β-Glucuronidase *mµMol freies* Phenolphthalein/Std. Malat Dehydrogenase *mµ*Mol oxyd. $NADH_2$/min Protein mg/10⁷ Zellen t Test Normalpersonen gegen die verschiedenen Patientengruppen

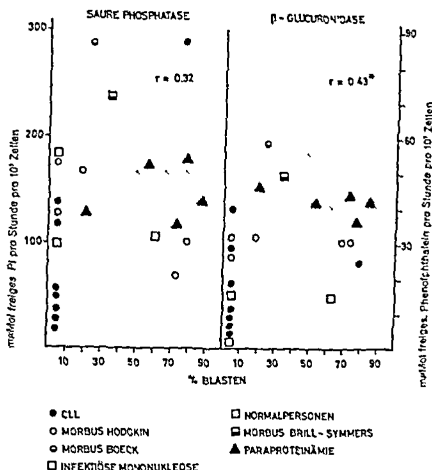


Abb. 2 Beziehung zwischen der Aktivität der sauren Phosphatase und β-Glucuronidase in unstimulierten Lymphozyten von Patienten mit lymphoretikulären Erkrankungen und der Blastentransformation nach 5tägiger Inkubation mit PHA r = Korrelationskoeffizient, * = signifikant gegen Null.

die in Lymphozytenhomogenaten der untersuchten Normalpersonen und der Patienten mit lymphoretikulären Erkrankungen gemessen wurden. Ausserdem sind die Transformationsraten nach 3-, 5- oder 7 tägiger Inkubation mit PHA aufgeführt. In Kulturen normaler Lymphozyten wurden am 3. Tag 53,6±8,6% transformierte Zellen und 4,6±2,3‰ Mitosen festgestellt; eine weitere signifikante Zunahme der Blastenzahl wurde am 5. und 7. Tag vermisst. In Homogenaten unstimulierter CLL-Lymphozyten wurden signifikant niedrigere Aktivitäten von β-Glucuronidase als in entsprechenden Präparationen

normaler Zellen bestimmt. Die Aktivitäten der sauren Phosphatase waren nicht einheitlich. Zellen von CLL-Patienten mit hohen peripheren Lymphozytenzahlen (über 40000 µl) ließen eine signifikante Verminderung dieser Enzymaktivität erkennen, während bei Patienten mit nur leichter Lymphozytose unauffällige saure Phosphatase-Aktivitäten beobachtet wurden. Die mittlere Aktivität der MDH und der mittlere Proteingehalt zeigten keine signifikanten Abweichungen von der Norm. Von einer Ausnahme abgesehen waren die Transformationsraten und die Zahl der Mitosen nach Zusatz von PHA im Vergleich zum Normalkollektiv sehr stark vermindert. Die in unstimulierten Hodgkin-Lymphozyten bestimmten Enzymaktivitäten und der Proteingehalt dieser Zellen stimmten mit den Normwerten überein oder waren erniedrigt. Die Transformationsraten waren nicht einheitlich. Drei Patienten im Stadium der Generalisation zeigten nach 3 Tagen deutlich niedrigere Werte als 2 Patienten mit einer weitgehend lokalisierten Form der Erkrankung. In den von Paraproteinämiepatienten gewonnenen Lymphozytenhomogenaten war der Proteingehalt höher als in entsprechenden Präparationen normaler Lymphozyten. Bei weitgehend normaler Aktivität der Enzyme/Zelle resultierte daraus eine Erniedrigung der Enzymaktivitäten mg Zellprotein (spezifische Aktivität), die jedoch nur bei der β-Glucuronidase und der MDH signifikant war. Die Transformationsraten lagen nach 3 Tagen bei 4 von 5 Patienten im Normbereich, 1 Patient zeigte zu diesem Zeitpunkt deutlich erniedrigte Werte. Im Gegensatz zu den Paraproteinämie-Lymphozyten ließ sich in den Lymphozytenhomogenaten der Patienten mit infektiöser Mononukleose eine signifikante Erniedrigung der β-Glucuronidase- und MDH-Aktivität sowie des Proteingehaltes nachweisen, während die Aktivität der sauren Phosphatase weniger stark vermindert oder normal war. Bei 2 Patienten waren die Transformationsraten deutlich reduziert, während eine Patientin, die sich in der spätesten Krankheitsphase befand, bereits am 2. Tag normale Transformationswerte bot. Bei deutlich gesteigerter Aktivität der sauren Phosphatase und erhöhtem Proteingehalt der Homogenate zeigten Lymphozyten einer Patientin mit grossfollikulärem Lymphoblastom nach 3 Tagen eine verminderte Transformationsrate. Analoge Veränderungen der sauren Phosphatase und des Proteingehaltes sowie eine Erhöhung der MDH-Aktivität fanden sich in den Lymphozyten eines Patienten mit Morbus Boeck. In diesem Fall war die Transformationsrate nach 3 Tagen ebenfalls vermindert.

Diskussion

Um einen Zellverlust während der Präparation zu vermeiden, wurden die Lymphozyten bei den hier beschriebenen Versuchen durch mehrmaliges Frieren und Tauen in den Kulturgläsern homogenisiert. Bei früheren Untersuchungen [7] war die Homogenisation von normalen und CLL-Lymphozyten dagegen mit einem Glashomogenisator vom Potter-Elvehjem-Typ erfolgt. Ein Vergleich der Ergebnisse beider Versuchsreihen zeigt, dass die Aktivität der lysosomalen Enzyme saure Phosphatase und β-Glucuronidase in Homogenaten, die durch Frieren und Tauen gewonnen wurden, höher war als in Homogenaten, die mit einem Potter-Homogenisator hergestellt wurden. Diese Abweichungen konnten bei normalen und CLL-Lymphozyten in gleicher Weise nachgewiesen werden. Da alle Homogenate mit Triton X-100 behandelt und anschliessend bei 20000 *g* × 10 min zentrifugiert wurden, darf angenommen werden, dass die in Lysosomen lokalisierten Enzyme in dem für die biochemische Analyse verwendeten Überstand in löslicher Form quantitativ vorlagen und daher für die beobachteten Aktivitätsunterschiede von saurer Phosphatase und β-Glucuronidase nicht verantwortlich gemacht werden können [23]. Weitere Versuche müssen klären, ob bei dem kryolytischen Homogenisationsvorgang Aktivitäten von saurer Phosphatase und β-Glucuronidase direkt oder durch den anschliessenden Zusatz von Triton X-100 unsedimentierbar (bei 20000 *g* × 10 min) gemacht werden, die nicht in Lysosomen lokalisiert, sondern mit anderen Zellbestandteilen assoziiert sind. Im Gegensatz zur Aktivität der lysosomalen Enzyme war der Proteingehalt im 20000 *g* × 10 min-Überstand nach Kryolyse geringer als nach Potter-Homogenisation. Aus diesen Befunden geht hervor, dass Ergebnisse, die mit beiden Homogenisationsverfahren gewonnen werden, nicht direkt vergleichbar sind.

Erwartungsgemäss reagierten normale Lymphozyten auf PHA 3 Tage nach Stimulierungsbeginn mit einer maximalen Blastentransformation und Mitoseaktivität. Dagegen zeigten Lymphozyten von *Patienten mit lymphoretikulären Erkrankungen* zu diesem Zeitpunkt ein uneinheitliches Stimulierungsbild.

Bei Morbus Hodgkin entsprachen die Ergebnisse den Befunden von Havemann [18], der mit zunehmender Generalisation der Erkrankung eine Abnahme der Frühreaktion (3. Tag) beobachtete. In diesen Fällen wurde das Reaktionsmaximum häufig erst am 5. bzw.

7 Tag erreicht. An einem grossen Krankengut fanden HAN und SOKAL [15a] dass das Ausmass der Blastentransformation nach 5 tägiger Stimulierung mit PHA weniger mit dem Stadium der Erkrankung als mit den therapeutischen Massnahmen korrelierte Dabei ging eine Strahlentherapie mit einer stärkeren Verminderung der Lymphozyten reaktion auf PHA einher als eine zytostatische Behandlung SALMON und FUDENBERG [43] stellten beim Plasmozytom und der Makroglobulinämie Waldenstrom nach 3 tägiger Stimulierung mit PHA stets eine erhebliche Verminderung des Einbaus von Nukleinsäurepräkursoren in die Lymphozyten fest Übereinstimmende Ergebnisse wurden von DOUGLAS *et al* [12] nur bei unbehandelten Plasmozytompatienten nicht jedoch bei der Makroglobulinämie Waldenstrom erhalten Bei den hier mitgeteilten Untersuchungen die mit morphologischer Methode durchgeführt wurden reagierten Lymphozyten von Patienten mit Makroglobulinämie Waldenström und behandeltem Plasmozytom normal auf PHA wie dies auch von ASTALDI *et al* [2] beschrieben wurde Die bei dem unbehandelten Plasmozytompatienten verifizierte Spätreaktion lässt sich analogen Befunden von DOUGLAS *et al* [12] zuordnen Die Patienten mit infektiöser Mononukleose bei denen die Untersuchung innerhalb der ersten 3 Wochen nach Beginn der klinischen Symptomatik vorgenommen wurde zeigten unabhängig von der Zahl atypischer mononukleärer Elemente im peripheren Blut eine reduzierte PHA Reaktivität der kleinen Lymphozyten ein Befund der mit den von RUBIN [41] mitgeteilten Ergebnissen vergleichbar ist Dagegen waren die Lymphozyten der Patientin die sich in der 5 Krankheitswoche befand regelrecht stimulierbar Die Lymphozyten von je einem Patienten mit Morbus Brill Symmers und Morbus Boeck wiesen eine abgeschwachte Fruhreaktion auf liessen jedoch eine gute Spatreaktion erkennen

In den unstimulierten Lymphozyten der Patienten mit den besprochenen Erkrankungen war der Gehalt an saurer Phosphatase und β Glucuronidase unterschiedlich Beide Enzyme waren bei Paraproteinämien Morbus Brill Symmers und Morbus Boeck annähernd normal oder sogar deutlich erhoht Dagegen fand sich bei allen untersuchten Fallen von Morbus Hodgkin und infektioser Mononukleose eine Verminderung der β-Glucuronidase Aktivität ohne dass in jedem Fall eine parallele Reduktion der Aktivität der sauren Phosphatose festzustellen war Im Gegensatz zu den hier mitgeteilten Ergebnissen wiesen YAM und MITUS [52] bei Morbus Hodgkin und infektioser

Mononukleose stets regelrechte *β*-Glucuronidase-Aktivitäten nach. Dieser Unterschied kann methodisch bedingt sein, da die genannten Autoren die Enzymaktivitäten nicht bio-, sondern zytochemisch bestimmten.

Sieht man von der infektiösen Mononukleose ab, so gingen mit den Normabweichungen der sauren Phosphatase und *β*-Glucuronidase keine analogen Veränderungen des nicht lysosomalen Enzyms MDH einher. Diese Beobachtung ist mit der Annahme vereinbar, dass der Gehalt an lysosomalen Enzymen in Lymphozyten spezifisch reguliert wird.

In unstimulierten Paraproteinämie-Lymphozyten war der Proteingehalt im Vergleich zu normalen Zellen auffallend hoch. Über Art und Herkunft dieses Proteins geben die vorliegenden Untersuchungen jedoch keinen Aufschluss.

Bei 7 von 8 untersuchten CLL-Patienten liessen die Lymphozyten 3 Tage nach PHA-Zusatz eine nur geringe Blastentransformation erkennen. Davon zeigten 3 Fälle eine Spätstimulierung nach 5–7 Tagen. Die von mehreren Autoren [4, 16, 35, 38, 42a, 46] beobachtete negative Korrelation zwischen der Reaktion auf PHA und der Höhe der peripheren Lymphozytenzahl bestätigte sich auch in dem vorliegenden kleinen Kollektiv, da in Zellkulturen von Patienten, deren Lymphozytenzahl etwa 100000/μl betrug oder überschritt, keine stärkere Früh- oder Spätstimulierung eintrat.

Aus den Ergebnissen der biochemischen Untersuchungen geht hervor, dass eine negative Korrelation auch zwischen dem *β*-Glucuronidase-Gehalt der unstimulierten CLL-Zellen und dem Grad der Blutlymphozytose besteht, was bereits von Yam und Mitus [52] aufgrund von zytochemischen Untersuchungen diskutiert wurde. Es ist zu erwarten, dass durch die Untersuchung einer grösseren Patientenzahl auch für die saure Phosphatase eine derartige Beziehung statistisch gesichert werden kann.

Unsere Daten stützen die eingangs erwähnte Annahme, dass bei der CLL im peripheren Blut 2 oder mehr Lymphozytenpopulationen vorkommen, deren prozentualer Anteil an der Gesamtzellzahl nicht konstant ist und sich mit der peripheren Lymphozytenzahl ändert [4, 35]. Funktionell unterschiedliche Lymphozytenpopulationen wurden von Havemann [18] auch für den Morbus Hodgkin und von Douglas *et al.* [12] für die Paraproteinämien postuliert. Darüber hinaus sprechen neuere Untersuchungen dafür, dass bei Gesunden

ebenfalls eine Inhomogenität der Blutlymphozyten besteht So zeigen z B Blutlymphozyten einen unterschiedlichen Stimulierungsablauf nach Zusatz verschiedener unspezifischer Mitogene [12], ausserdem reagieren Zellen, die im Dichtegradienten getrennt wurden, uneinheitlich auf PHA [40] Es stellt sich daher die Frage ob bei der CLI tatsachlich «pathologische» Zellen auftreten die bei Gesunden vermisst werden oder ob nur eine oder mehrere Lymphozytenpopulationen stark vermehrt sind, die normalerweise nur einen geringen Prozentsatz der Blutlymphozyten ausmachen

Die besprochenen Befunde deuten an, dass möglicherweise die CLL-Lymphozyten, die eine Verminderung der lysosomalen Enzyme saure Phosphatase und β-Glucuronidase aufweisen, mit den Zellen identisch sind deren Reaktion auf PHA gestort ist Eine derartige Identität könnte ihrerseits vermuten lassen, dass die Enzymverminderung in unstimulierten Lymphozyten von kausaler Bedeutung fur die reduzierte PHA Stimulierbarkeit ist Voraussetzung dafür wäre allerdings eine strenge, positive Korrelation zwischen beiden Parametern die nicht nur bei der CLL, sondern auch bei allen anderen lymphoretikulären Erkrankungen mit verminderter PHA Reaktivitat nachweisbar sein musste Die Resultate unserer Untersuchungen zeigen eindeutig dass eine derartige Korrelation fur die saure Phosphatase nicht besteht Der zwischen der β Glucuronidase Aktivität und der Blastentransformation gefundene niedrige Korrelationskoeffizient schliesst auch für dieses Enzym eine strenge Korrelation aus Aufgrund der Ergebnisse der erwähnten zytochemischen Untersuchungen an Lymphozyten von Patienten mit CLL und anderen lymphoretikularen Erkrankungen diskutierten YAM und MITUS [52] zwar eine Korrelation zwischen dem Enzymgehalt und der PHA induzierten Blastentransformation, betonten aber, dass diese Korrelation nicht «absolut» war und immer wieder Ausnahmen beobachtet wurden

Es kann somit angenommen werden dass die Fähigkeit von Lymphozyten zu einer regelrechten Fruhreaktion auf PHA nicht an das Vorhandensein einer normalen Gesamtaktivität der lysosomalen Enzyme saure Phosphatase und β-Glucuronidase gebunden ist Es ist allerdings bisher unbekannt, ob in Lysosomen von Lymphozyten mit herabgesetzter Stimulierbarkeit die Aktivitat anderer Enzyme vermindert ist und/oder qualitative Enzymveranderungen bestehen Darüber hinaus konnten in derartigen Zellen Mechanismen gestort sein, die die Verfugbarkeit und/oder Aktivierung lysosomaler Enzyme be

einflussen. Möglicherweise stellt die initiale Lysosomenlabilisierung, die in normalen Lymphozyten nach PHA-Zusatz nachweisbar wird [21], einen derartigen Mechanismus dar. Diese Hypothese wird dadurch gestützt, dass die bisher an CLL-Lymphozyten mit gestörter Reaktion auf PHA durchgeführten Untersuchungen keinen Anhalt für eine Lysosomenlabilisierung erbrachten [7]. Ob eine Lysosomenlabilisierung auch in Lymphozyten von Patienten mit anderen lymphoretikulären Erkrankungen vermisst wird, ist noch nicht geklärt.

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Zusammenfassung

In unstimulierten Lymphozyten von Normalpersonen und Patienten mit chronischer lymphatischer Leukämie (CLL), Morbus Hodgkin, Paraproteinämie (Plasmozytom, Makroglobulinämie Waldenström), infektiöser Mononukleose, Morbus Brill-Symmers und Morbus Boeck wurden die Aktivitäten der lysosomalen Enzyme saure Phosphatase und β-Glucuronidase biochemisch bestimmt. Die Aktivitäten der sauren Phosphatase und/oder β-Glucuronidase waren bei einem Teil der Fälle von CLL, bei der infektiösen Mononukleose und beim Morbus Hodgkin erniedrigt, während sie bei den übrigen Erkrankungen normal oder erhöht waren. Im Gegensatz zu normalen Lymphozyten zeigten CLL-Lymphozyten und Lymphozyten von Patienten mit generalisiertem Morbus Hodgkin nach 3tägiger Inkubation mit PHA fast immer eine stark reduzierte Blastentransformation, während das Stimulierungsbild bei den anderen Erkrankungen sehr unterschiedlich war. Zwischen dem Gehalt der unstimulierten Lymphozyten an den lysosomalen Enzymen saure Phosphatase und β-Glucuronidase und dem Ausmass der nach 3 Tagen nachweisbaren Reaktion auf PHA bestand keine enge Korrelation.

Summary

Activities of lysosomal enzymes acid phosphatase and β-glucuronidase were determined in purified unstimulated peripheral blood lymphocytes from normal subjects and from patients with chronic lymphocytic leukemia (CLL), Hodgkin's disease, paraproteinemia (multiple myeloma, Waldenström's macroglobulinemia), infectious mononucleosis, giant follicular lymphoma (Brill-Symmers) and sarcoidosis. Activities of acid phosphatase and/or β-glucuronidase were diminished in most cases of CLL as well as in several cases of Hodgkin's disease and infectious mononucleosis but proved to be normal or elevated in lymphocytes derived from the other diseases under study. After 3 days of incubation with phytohemagglutinin (PHA) lymphocytes from most patients with CLL, patients with advanced Hodgkin's disease and some patients with the other lymphoreticular disorders showed an impaired blast transformation as compared to normal lymphocytes. No close correlation

was found between the total activities of the lysosomal enzymes acid phosphatase and β-glucuronidase present in unstimulated lymphocytes and the degree of blast transformation after 3 days of incubation with PHA.

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DNS-Synthese in Blut-Lymphozyten beim malignen Lymphogranulom[1]

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Eine Vermehrung atypischer, mononukleärer Zellen wurde bei zahlreichen, ätiologisch unterschiedlichen Erkrankungen, wie viralen und bakteriellen Infekten [1, 2, 3, 4], beim malignen Lymphogranulom [4, 5], bei Autoaggressionserkrankungen [4, 6], refraktären Anämien [6], Drogenuberempfindlichkeit [1], nach Immunisierung [4] und Ganzkorperbestrahlung [7] beschrieben

Durch autoradiographische und zytophotometrische Untersuchungen konnte gezeigt werden, dass sich ein Teil dieser Zellen in DNS-Synthese befindet [1–7]. Auf Grund des zytochemischen Verhaltens [8, 9], der Ultrastruktur [10] und der fehlenden Fähigkeit zur Phagozytose [4] scheint die Zuordnung der atypischen, mononukleären Zellen zur lymphatischen Reihe gerechtfertigt, der Ort ihrer Bildung und ihre Funktion sind nach wie vor unklar und umstritten

In der vorliegenden Arbeit wurde geprüft, ob Beziehungen zwischen der Zahl der DNS-synthetisierenden Lymphozyten und den klinischen Parametern beim malignen Lymphogranulom bestehen

Material und Methoden

Patienten An 26 Patienten mit histologisch gesichertem malignem Lymphogranulom wurden insgesamt 43 Beobachtungen durchgeführt 5 der Patienten waren unter 21 Jahre

[1] Die vorliegenden Untersuchungen wurden mit Unterstutzung des Fonds »Kampf dem Krebs« durchgeführt

alt, 6 zwischen 22 und 31, 8 zwischen 32 und 41 Jahren und 8 über 42 Jahre alt. 14 dieser 26 Patienten befanden sich im Stadium der lokalisierten Erkrankung (Stadium I and II) und 12 im Generalisations-Stadium (Stadium III und IV) (Stadieneinteilung nach der Rye-Klassifikation [11]). Allgemeinsymptome wie Fieber über 37,5°C, BSG über 50 mm in der ersten Stunde, Hb unter 11 g%, Leukozytose mit pathologischer Linksverschiebung bestanden bei 7 Patienten mit lokalisierter und 8 Patienten mit generalisierter Form der Erkrankung. 8 der 26 Patienten waren noch völlig unbehandelt, 16 Patienten wurden innerhalb von 12 Monaten vor oder während der Untersuchungszeit mit Zytostatika (Endoxan, Natulan oder Velbe – 6 Patienten) oder Strahlentherapie (10 Patienten) behandelt, bei 2 weiteren Patienten lag die letzte Strahlenbehandlung länger als 12 Monate zurück.

Kontrollen. 7 gesunde Kontrollpersonen wurden ausgewählt, bei denen keine klinisch manifesten Infektionen vorlagen.

Zellkultivierung. 20 ml venöses Blut wurde mit 0,15 ml kresolfreiem Heparin (10000 IE/ml, Hämoderivate, Wien) steril abgenommen und die Erythrozyten spontan oder durch Zusatz von 1/5 Vol 3% Dextran durch 30 min bei 37°C sedimentiert. Der leukozytenreiche Überstand wurde zu gleichen Teilen mit Kulturmedium TC 199 (Burroughs Wellcome, London) verdünnt und in 25 ml Kulturflaschen angesetzt.

[illegible]

gestrichen und 12 h in 96% Äthanol fixiert.

Autoradiographie. Von alkoholfixierten Präparaten wurden Dipping Autoradiogramme [illegible]

[illegible] Zellen ausgewertet und der Mittelwert errechnet.

Kombinierte Autoradiographie – Zytophotometrie. Markierte, alkoholfixierte Zellstriche wurden nach Feulgen gefärbt, photographiert und der relative DNS-Gehalt mit einem integrierenden Mikrodensitometer nach Deeley (GN 2, Fa. Barr & Stroud, Glasgow) bei einer Wellenlänge von 560 nm bestimmt. Anschließend wurden die Präparate autoradiographiert und die Ergebnisse der Autoradiographie mit denen der Zytophotometrie über die Photographien korreliert (Methode [12]).

Mitoseindex. Von 4 Patienten mit malignem Lymphogranulom und 7 Patienten mit [illegible]

[illegible] pro Patient ausgewertet.

Zytochemie. Durch gleichzeitigen zytochemischen Nachweis der Naphthol AS- und Naphthol AS-D-Chloracetat Esterase (Methode [13]) liessen sich Monozyten und Granulozyten färberisch verschieden darstellen. Lymphozyten blieben weitgehend ungefärbt. Anschliessend wurden die gefärbten Striche autoradiographisch ausgewertet und je 100 ³H-TdR markierte Zellen von 5 Lymphogranulom Patienten auf ihr färberisches Verhalten geprüft.

Statistik. Die Prüfung der Mittelwertsunterschiede erfolgte mittels des Studentschen t-Test.

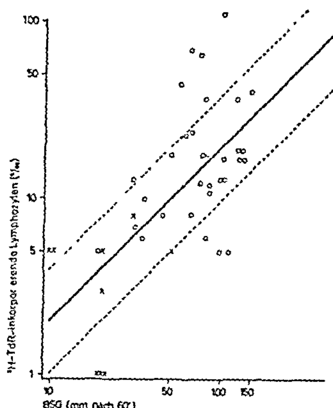


Abb 1 Beziehungen zwischen der Anzahl ³H-TdR-inkorporierender Lymphozyten (‰) und der BSG, 43 Einzelbeobachtungen an 26 Lymphogranulom Patienten (o), davon 8 Patienten in Remission (x) Gleichung der Geraden $\log y = -0{,}6950 + 1{,}0004 \log x$ Sayx = ± 0,310, r = 0,6712, P < 0,001

Ergebnisse

Beziehungen zwischen der Anzahl DNS-synthetisierender Lymphozyten und klinischen Parametern beim malignen Lymphogranulom. Bei Lymphogranulom-Patienten fanden wir im Mittel 18,4±3,2‰ ³H-TdR-inkorporierende Lymphozyten, während dieser Index bei der Kontrollgruppe 1,33±0,28‰ betrug Der Unterschied zu den Normalen war hoch signifikant (P <0,001).

Während die Zahl DNS-synthetisierender Blutlymphozyten mit dem Stadium der Erkrankung nicht korreliert, fanden sich bei Patienten mit den Symptomen einer Allgemeinerkrankung (Definition siehe Methodik) signifikant höhere Werte ($\bar{x}$=25,5±4,6‰, 14 Beobachtungen) als bei Patienten ohne solche ($\bar{x}$=4,6±0,6‰, P <0,005,

28 Beobachtungen). 8 noch völlig unbehandelte Patienten zeigten signifikant höhere Werte ($\bar{x}=38,3\pm12,7$‰) als bereits ein- oder mehrfach behandelte ($n=35$, $\bar{x}=14,5\pm2,4$‰; $P<0,005$). Unter diesen zeigten Patienten in Remission nach erfolgreicher Therapie besonders niedrige Indices ($n=9$, $\bar{x}=3,8\pm1,0$‰). Während und unmittelbar nach Behandlung wurde jedoch auch dann eine Erniedrigung der Werte beobachtet, wenn keine Besserung erzielt werden konnte, diese Erniedrigung war jedoch im Vergleich zu den Remissionen nicht so ausgeprägt.

Eine besonders deutliche Beziehung bestand zwischen dem Prozentsatz ^{3}H-TdR-inkorporierender Blutlymphozyten und den serologischen Entzündungszeichen, insbesondere der BSG. Diese Korrelation zwischen der Zahl DNS-synthetisierender Lymphozyten und der BSG war hoch signifikant ($r=0,6712$; $P<0,001$). Bei den Patienten in Remission waren dieser Index und die BSG vergleichbar erniedrigt (Abb. 1). Ebenso lag die Zahl DNS synthetisierender Lymphozyten bei Patienten mit einer negativen CRP-Reaktion ($n=8$, $\bar{x}=5,0\pm1,5$‰) signifikant unter den Werten der Patienten mit positiver Reaktion ($n=18$, $\bar{x}=27,8\pm5,9$‰, $P<0,01$). Ähnliche Unterschiede ergaben sich auch bei Gegenüberstellung der Patienten mit weitgehend normaler bzw. deutlich pathologischer Elektrophorese (Alpha 2 >9%, Gamma >23%). Zwischen dem Prozentsatz oder der absoluten Zahl DNS synthetisierender Lymphozyten und dem Stadium der Erkrankung, der Krankheitsdauer, den Leukozyten-, Lymphozyten-, Monozyten- und Eosinophilenzahlen sowie der Art der durchgeführten Behandlung bestand keine Beziehung. Der höchste Prozentsatz ^{3}H-TdR-markierter Zellen war bei Patienten unter 31 Jahren nachweisbar. Indices über 30‰ wurden bei 4 der 11 Fälle unter dem 31. Lebensjahr, aber bei keinem der 15 Fälle mit höherem Alter beobachtet. Dieser Unterschied konnte jedoch statistisch nicht gesichert werden.

Mitoseindices. Bei 4 Lymphogranulom-Patienten und 7 Patienten mit Reizlymphozytose im Rahmen von Infekten (Näheres siehe Methodik) wurden nach 6stündiger Colchicinblockierung die Mitoseindices bestimmt. Mit den ^{3}H-TdR-Markierungsindices nach 1 h und einem geschätzten Verhältnis der Synthese- zur Mitosedauer von 1:12 [14] wurde die erwartete Mitosehäufigkeit unter der Annahme eines ungestörten Zellstromes von der Synthese- in die Mitosephase errechnet und den gefundenen Werten gegenübergestellt. Wie Abbildung 2 zeigt, lagen die gefundenen Mitosehäufigkeiten ausnahmslos

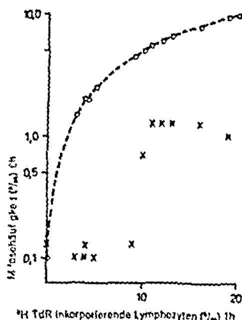


Abb. 2 Erwartete (o) und gefundene (x, *) Mitosehäufigkeit im Verhältnis zur Zahl ³H-TdR-*inkorporierender Lymphozyten* (‰). *Die* erwartete Mitosehäufigkeit wurde aus der beobachteten Zahl DNS-synthetisierender Lymphozyten nach 60 min ³H-TdR Markierung und einem angenommenen Verhältnis der Synthese zur Mitosedauer von 1 : 12 [14] errechnet. Als gefundene Mitosehäufigkeit wurde die Zahl der Teilungsfiguren (‰) nach *6stündiger Colchicinzugabe* (1×10^{-6} M) eingetragen. * = *Lymphogranulom* Patienten x = Patienten mit Infekten (näheres s. Methodik)

etwa eine Zehnerpotenz unter den erwarteten Werten. Zwischen Lymphogranulom-Patienten und Patienten mit Infektionen fanden sich in dieser Hinsicht keine Unterschiede.

Kombinierte Zytophotometrie-Autoradiographie. Eine Kombination von zytophotometrischer Bestimmung des relativen DNS-Gehaltes mit der Autoradiographie nach ³H-TdR-Markierung ermöglicht eine Einordnung unmarkierter Zellen in die G_1- bzw. G_2-Phase. Typischerweise sind Zellen in der G_1-Phase durch einen relativen DNS-Gehalt um 2 n und fehlenden Einbau von ³H-TdR gekennzeichnet, solche in der DNS-Synthesephase zeigen einen DNS-Gehalt zwischen 2 und 4 n und sind mit ³H-TdR markiert, während die Zellen in der G_2-Phase unmarkiert bleiben und ebenfalls einen DNS-Gehalt um 4 n zeigen. Insgesamt wurden 870 Zellen von 3 Lymphogranulom-Patienten ausgewertet. Wie Abbildung 3 zeigt, fanden sich alle unmarkierten Zellen im Bereich des G_1-Gipfels, es liessen sich keine sicheren G_2-Zellen ab-

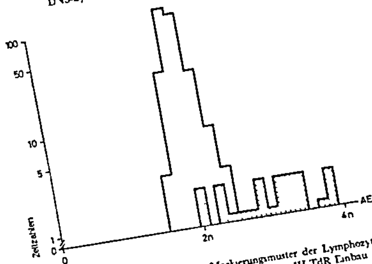


Abb. 3. Relativer DNS-Gehalt und ^{3}H-TdR-Markierungsmuster der Lymphozyten eines repräsentativen Lymphogranulom-Patienten. Dunkle Felder = ^{3}H-TdR-Einbau.

grenzen. Zellen mit einem relativen DNS-Gehalt zwischen 2 und 4 n waren ausnahmslos markiert. Es fanden sich keine Zellen mit einem DNS-Gehalt von über 4 n.

Kombinierte Zytochemie – Autoradiographie und Morphologie. Durch zytochemische Darstellung der Naphthol-AS-Azetat- und der Naphthol-AS-D-Chlorazetat-Esterase am selben Ausstrich liessen sich Monozyten und Granulozyten verschieden anfärben, Lymphozyten blieben weitgehend ungefärbt [13]. Bei Kombination von Autoradiographie und Zytochemie fanden sich unter je 100 ^{3}H-TdR-inkorporierenden Zellen keine, die durch die Färbung als monozytäre oder granulozytäre Zelle gekennzeichnet waren (5 Fälle).

In panoptischen Färbungen stellte sich die Mehrzahl DNS-synthetisierender Lymphozyten (ca. 70%) als grosse Zellen (16–25 μ) mit reichlichem, lichtblau bis intensiv basophil gefärbten Zytoplasma dar, welches häufig Vakuolen enthielt. Die Zellkerne waren meist oval, leicht eingedellt, und häufig war eine deutliche perinukleare Aufhellung nachweisbar. Plasmozytoide Zellen sowie typische grosse Lymphozyten waren nur selten markiert, markierte kleine Lymphozyten wurden bei Durchmusterung der zahlreichen Präparate zwar gesehen, stellten jedoch eine ausgesprochene Rarität dar.

Diskussion

Bei der Untersuchung einer grösseren Gruppe von Patienten mit malignem Lymphogranulom fanden wir im Mittel eine Erhöhung des Anteils DNS-synthetisierender, zirkulierender Lymphozyten auf das etwa 15fache der Kontrollpersonen. Unsere Ergebnisse bei Normalpersonen stimmen gut mit den Befunden anderer Autoren überein. So fanden Epstein [3] sowie Crowther [4] bei gesunden Kontrollpersonen 0–2‰ markierte Lymphozyten, während unser Mittelwert 1,3±0,3‰ betrug.

Die Aktivität der Hodgkinschen Erkrankung und ihr Ausbreitungsgrad waren von unterschiedlichem Einfluss auf die Zahl DNS-synthetisierender Blutlymphozyten. Ihr Prozentsatz stand in deutlicher Beziehung zu den entzündlichen Allgemeinsymptomen. Bei Patienten mit akuten Krankheitszeichen und entsprechenden Symptomen (z.B. Anämie, Fieber, BSG-Beschleunigung) fanden wir etwa fünfmal höhere Werte als bei Patienten ohne solche Erscheinungen. Patienten im Remissions-Stadium zeigten dagegen niedrigere bis normale Indices. Das Ausmass des lymphogranulomatösen Organbefalles selbst war dagegen ohne offensichtlichen Einfluss auf die Vermehrung dieser Zellen; auch bei lokalisierten Erkrankungen mit Allgemeinreaktion konnten hohe Markierungsindices beobachtet werden.

Bei den markierten Zellen handelte es sich vorwiegend um grosse Zellen mit einem breiten Zytoplasmasaum und ovalem bis nierenförmigem Kern. Durch zytochemische Methoden ist eine Differenzierung mononukleärer Zellen in solche der lymphatischen Reihe gegenüber Monozyten und ihnen verwandten Zellen erleichtert [13]. DNS-synthetisierende, zirkulierende Monozyten konnten von uns beim Morbus Hodgkin und einigen anderen Fällen mit Monozytose bisher nicht beobachtet werden, sie wurden auch bei Normalpersonen nicht gefunden [15].

DNS-synthetisierende Blutlymphozyten wurden vor allem bei infektiöser Mononukleose eingehend untersucht [2, 3, 6, 17]. Es war daher von Interesse, autoradiographische u[illegible] [illegible]hotometrische Befunde dieser Zellen [illegible] gegen[illegible] [illegible] Beide Zellen waren morpholo[illegible] Auch beim [illegible] [illegible]gkin ergaben sich keine Hinweis[illegible] [illegible] zirkul[illegible] [illegible]ider Zellen. Bei beide[illegible] wurde ein [illegible] zwischen dem Zellanteil in D[illegible] dem in [illegible] [3, 17].

Weniger als insgesamt 10 Mitosen wurden beim Auszählen von über 100000 Lymphozyten unserer Patientengruppe gefunden, die etwa 2000 DNS-synthetisierende Zellen enthielten (die erwartete Mitosehäufigkeit wurde dagegen (Methodik [14]) etwa 200 Mitosen betragen). Als mögliche Ursache der Diskrepanz zwischen markierten Zellen und solchen in Zellteilung konnten eine extreme Verlängerung der DNS-Synthese-Dauer, ein Liegenbleiben von Zellen in der G_2-Phase oder ein Zelluntergang bzw. ihr Verlust aus der Zirkulation während oder nach der DNS-Synthese-Phase in Frage kommen. Gegen die erste Möglichkeit spricht, dass wir in Übereinstimmung mit Befunden von Epstein an Drüsenfieberzellen [3] vergleichbare Silberkornzahlen bei Blutlymphozyten von Hodgkin-Patienten und PHA-stimulierten Lymphozyten fanden, deren DNS-Synthese-Dauer im Bereich der meisten Zellen *in vivo* liegt [16]. Auch fanden wir analog den Befunden von Cooper an Mononukleosezellen [17] keine Hinweise für eine Anhäufung von Zellen in der G_2-Phase. Wie Cooper *et al.* weiter zeigten, treten diese spontan DNS-synthetisierenden Lymphozyten auch in Kurzkulturen nur vermindert in die Mitose ein. Auch wir beobachteten in Kulturen mit Colchicin-Zusatz Mitosehäufigkeiten, die weit unter den erwarteten Werten lagen. Diese Befunde stehen jedenfalls im Gegensatz zu *in vitro*-Beobachtungen an Blutlymphozyten, die unter der Wirkung verschiedener Mitogene (z.B. Phytohämagglutinin) in die DNS-Synthese eintreten und dann auch in höherer Zahl Mitosefiguren zeigen. Die Ergebnisse dieser *in vitro*-Versuche machen es daher wenig wahrscheinlich, dass ein Verschwinden dieser Zellen nach der DNS-Synthese aus dem Kreislauf (z.B. Ansiedlung im lymphatischen Gewebe) für das beobachtete Phänomen allein verantwortlich ist. Sie legen nahe, an ein Zugrundegehen solcher Zellen als zusätzlichen Mechanismus zu denken.

Es ist gut gesichert, dass Blutlymphozyten bei vielen Patienten mit Morbus Hodgkin, insbesondere im anergischen Stadium, eine verminderte [18] oder zumindest verzögerte [19] Blastentransformation in Gegenwart von PHA zeigen. Nach unseren Ergebnissen (unveröffentlicht) war eine erhöhte Anzahl DNS-synthetisierender Zellen auch bei solchen Lymphogranulom-Patienten nachweisbar, die in der PHA-Kultur nach 72 h eine hochgradig verminderte Transformation zeigten. Stark erhöhte Zahlen DNS-synthetisierender Blutlymphozyten wurden schliesslich auch dann beobachtet, wenn bei der Hautallergie-Testung eine Anergie festgestellt wurde [4].

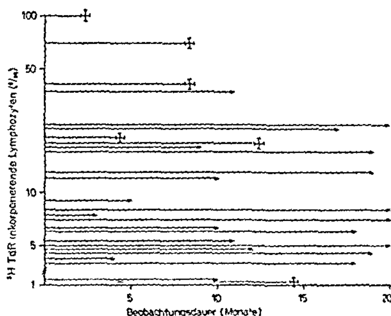


Abb. 4. Anzahl ^{3}H-TdR-inkorporierender Lymphozyten (‰) am Beginn der Verlaufsbeobachtung von 26 Lymphogranulom-Patienten sowie die Zahl der Todesfälle innerhalb von maximal 20 Monaten. † = verstorben. → = unter weiterer Beobachtung.

Bei Verlaufsbeobachtungen unserer Patienten ergaben sich suggestive Hinweise, dass ein hoher Markierungsindex prognostisch als ungunstiges Zeichen erschien. So sind von 9 Patienten mit einem Index über 15‰ 5 Patienten verstorben, während dies bei 17 Kranken mit niederigeren Werten nur einmal beobachtet wurde. Die Ergebnisse sind in Abbildung 4 zusammengestellt. Es bleibt abzuwarten, ob diese Befunde an grösseren Gruppen und nach längerer Beobachtungsdauer bestätigt werden können.

Wir möchten Herrn Dr. E. KNAPP für die statistische Auswertung und Frau URSULA MICHLMAYR für die technische Mitarbeit danken.

Zusammenfassung

Der ^{3}H-TdR-Einbau, der relative DNS-Gehalt und die mitotische Aktivität wurden an [illegible] Zahl DNS- [illegible] ontroll- [illegible] en der Erkrankung, jedoch nicht zum Ausmaß des lymphogranulomatösen Befalls. Patienten in einem Remissionsstadium zeigten niedrige bis normale Werte. Die Blutlymphozyten beim

malignen Lymphogranulom stellten eine euploide Zellpopulation dar. Im Verhältnis zur Anzahl DNS-synthetisierender Zellen wurden auffallend wenig Mitosen gefunden, ein Mißverhältnis, das auch nach Kurzkultivierung mit Colchicinzusatz gefunden wurde. Diese Beobachtung könnte durch ein vermehrtes Absterben dieser Zellen erklärt werden.

Summary

Investigations concerning ^3H TdR incorporation, DNA content and mitotic activity of circulating lymphocytes were carried out on 26 patients suffering from Hodgkin's Disease and 7 healthy controls. On the average the portion of DNA-synthesizing lymphocytes in Hodgkin's Disease was approximately 15 times that of the normals. A fairly close relationship between the numbers of lymphocytes in DNA-synthesis and the activity of the disease was observed. Whereas the extent of organ involvement had less effect on the number of labelled cells. Lymphocytes in patients with Hodgkin's Disease displayed an euploid DNA pattern. Although DNA-synthesising cells may represent up to 10% of the total number of peripheral lymphocytes, mitotic figure swere extremely rare and did not accumulate in the expected amount in short terme cultures, when colchicine was added to the medium.

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Glutathione Concentration and Stability of the Red Blood Cells in Acute Childhood Leukemia

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Anemia is so common in childhood leukemia that it might be considered an almost inevitable complication of the disease The pathogenesis of anemia is now viewed more as a dynamic process than as a crowding out of erythroid elements [1] Although in leukemia cellular abnormalities are by no means confined to leukocytes, little is known about the metabolism of red blood cells in this disease.

SABINE [2] and JESENOVEC *et al* [3] recently determined the concentration of erythrocyte glutathione in different kinds of adult leukemia, but no correlation has been made between relapse and remission values In this study GSH concentration and stability of red blood cells were determined in childhood leukemia in relapse and in remission

Materials and Methods

For control values blood was obtained from 25 apparently healthy subjects selected [illegible]

[illegible] were used to define relapse and remission [5] However, no routine repeated bone marrow examinations were made on patients in remission The GSH concentration and stability [illegible]

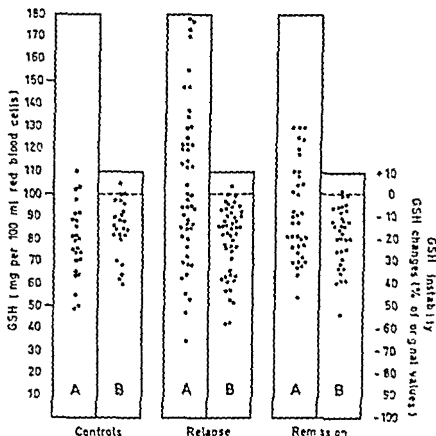


Fig 1 Erythrocyte GSH in controls and in acute stem cell leukemia in relapse and in remission (column A) and amount of GSH lost during instability experiments as per cent of original values (column B)

were measured by the cyanmethemoglobin technique Plasma GSH values were determined by BEUTLER's [f] method using the same amount of plasma instead of whole blood.

Results

The mean value for the control group was 78 8±3 1 mg (SD 15 7) GSH/100 ml erythrocytes The mean value of 49 determinations in 47 cases of acute stem cell leukemia in relapse was 103 35±5 11 mg (SD 35 82) Of 37 determinations in 27 patients in remission it was 91 86±3 47 mg (SD 21 17) The difference between these values was statistically significant $p<0.001$ between the relapse and control, and $p<0.02$ between the remission and control values (fig 1) The

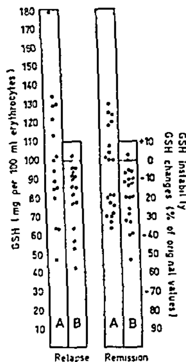


Fig 2 Red blood cell GSH (A) and amount of GSH lost during instability experiments (B) in relapse and remission of the same cases of acute childhood stem cell leukemia

relapse and remission values showed no statistically significant difference ($p > 0.05$)

In 16 of the acute stem cell leukemia cases relapse and remission values were both recorded on 16 and 20 occasions successively on the same patients (fig 2) In this group the mean values of GSH were 101.0 ± 7.92 mg (SD 31.7) per 100 ml red blood cell in relapse, and 93.65 ± 4.81 (SD 21.55) in remission Although the difference between relapse and remission was not significant ($p > 0.05$) both the values were again found to be significantly increased when compared to the control values

When glutathione instability was expressed as the amount of GSH lost during a 2 h incubation with acetylphenylhydrazine, the mean decrease was 12.48 ± 2.26 mg (SD 11.32) for the controls, $22.14 \pm$

Table I Erythrocyte GSH and the amount of GSH lost during instability experiments (per 100 ml red blood cells) in controls and in acute stem cell leukemia in relapse and remission

	Control (25 cases)	Relapse (49 determinations in 47 patients)	Remission (37 determinations in 27 patients)
GSH, mg	78 84±3 15 (SD±15 78)	103 35±5 11 (SD±35 82)	91 86±3 47 (SD±21 17)
GSH stability, difference, mg	12 48±2 26 (SD±11 32)	22 14±2 97 (SD±20 80)	17 32±2 42 (SD±14 74)

2 97 mg (SD 20 80) for patients in relapse, and 17 32±2 42 mg (SD 14 74) for those in remission. These values were not found to be different in the 16 patients on whom relapse and remission values were determined comparatively, being 18 81±4 26 mg (SD 17 06) for relapse and 18 80±3 60 mg (SD 16 16) for remission When the relapse and remission glutathione loss was compared both with control values and with each other, no difference was shown ($p > 0.05$ for each comparison) (fig 1 and 2 and table I) Erythrocyte G-6-PD was determined on the 3 patients in relapse, whose GSH values were lowest and whose glutathione was unstable One of these had also G-6-PD deficiency, but in the other two this enzyme activity was found to be normal, as were GSH values and glutathione stabilities during remission

In 5 patients with chronic myelocytic leukemia in relapse GSH values ranged from 99 to 176 mg per 100 ml erythrocytes In the cases of acute myelogenous leukemia, 4 relapse values were between 99 and 143 mg, and in 3 cases in remission it ranged from 72 to 108 mg per 100 ml erythrocytes

Discussion

Bernard and Mathé [9] were the first to report raised erythrocyte glutathione levels in patients with acute leukemia Although with the method they used the levels of total and reduced glutathione were much lower than our values, they also mentioned that before treatment red blood cell glutathione was increased

As was previously mentioned, Sabine [2] described elevated GSH values (over 100 mg/100 ml) in 4 out of 8 chronic myelocytic, 3 out

of 5 chronic lymphocytic and 4 out of 6 acute lymphatic leukemia cases in adults. JESENOVEC and FIŠER HERMAN [3] also found slightly increased GSH and oxydized glutathione in 22 adult myelogenous leukemia cases, but did not observe this in two cases of lymphatic leukemia. In the present study, 49 and 38% of the determinations were over 100 mg in relapse and in remission in acute stem cell leukemia cases respectively. One patient, who also had G 6-PD deficiency, was included in our statistical analysis, but in spite of this, the relapse and remission values were significantly higher than the control values.

The control values in this study are in general agreement with the findings of BEUTLER [7]. With the exception of the early newborn period, GSH values of the erythrocytes are not related to the age of the subjects [8, 11] or the age of the red blood cells [2, 10]. Our control values could be compared with those of the children with leukemia. Although some of the leukemic patients were fairly malnourished, we showed in a previous study that red cell GSH levels and GSH stability would not change with this condition [12].

Almost all the patients with acute leukemia in relapse had severe anemia. But none of them had iron deficiency anemia, in which GSH values are raised [11]. Because LAWRENCE [13] has shown that a low hematocrit can produce glutathione instability, hematocrit values were adjusted to over 20% in all cases by aspiration of plasma before the test was performed. In 2 patients with the lowest GSH levels without erythrocyte G 6-PD deficiency, GSH was unstable during relapse. However, their GSH stability was well within normal limits during subsequent remissions. Since neither low hematocrit nor constant agitation [14] were factors glutathione reductase deficiency, which is reported with acute leukemia, might explain the low GSH content and the instability of this tripeptide [15]. But we failed to assay this enzyme at this time.

It has been shown that concentration of GSH in leukocytes varies widely, and high erythrocyte GSH values could not be attributed to contamination with leukocytes, at least in chronic myelocytic leukemia [2]. Neither was any correlation seen between GSH levels and leukocyte counts in relapse or in remission in this material. Calculation of the correlation coefficient for X and Y = white blood cell count at the time of GSH determination in relapse and remission respectively gives a non significant value for both ($R_x = 0.1867$ and $R_y = 0.3129$, $p > 0.05$ for both). GSH values of the plasmas were determined only

in the cases with the highest erythrocyte GSH, and were found to be negligible.

Acute and chronic myelogenous leukemia cases were few for statistical evaluation, but erythrocyte GSH was above the normal value in almost every determination.

It should be emphasized that the method of glutathione determination indicated by BEUTLER *et al.* [6] is not specific for this tripeptide. We have also shown that cystein gives the same optical density changes of GSH [12]. In normal subjects this does not seem to be a handicap to the sensitivity of the method, because the erythrocyte-free amino acid level is very low. But it has been shown that 6-mercaptopurine decreases the amino acid incorporation of leukocytes in chronic myelogenous leukemia [16]. This cytotoxic drug was used in the treatment of most of our cases. Although most of the relapse values were obtained before treatment, the effect of this drug should be evaluated for free amino acid utilization and synthesis of GSH in the leukemic erythrocytes. This might explain the increase of GSH in relapse and in remission. It has been shown by LAWRENCE [17] that patients with megaloblastic *anemias have elevated red blood cell GSH levels.* Although megaloblastic changes were not observed in the initial bone marrow examinations, folic acid and vitamin B_{12} levels are not available which might be helpful in the explanation of this finding.

Acknowledgements. I am indebted to Prof. D. A. KUTSAL and Mr. H. R. KIVANC for the statistical evaluation. Dr. G. HIÇSÖNMEZ and Dr. B. YAMAK's help in the follow-up these patients is appreciated. I should also like to thank Miss S. MUTLU for her valuable technical assistance, and Mrs. E. DORAN for editorial assistance.

Summary

Erythrocyte glutathione (GSH) was determined in 70 cases of childhood leukemia. In acute stem cell leukemia significantly increased levels were found in 49 and 37 determinations in relapse and in remission respectively. With the exception of 3 patients, one of which had also G-6-PD deficiency, glutathione stability did not differ from cases in relapse and from controls. The GSH was also elevated in acute and chronic myelogenous leukemia.

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Fetal Erythropoiesis

Maturation in Megaloblastic (Yolk Sac) Erythropoiesis in the C 57 Bl/6 J Mouse[1]

(with colour plate I)

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The reticulocyte, a strictly morphologic definition based on vital staining, is currently thought to be an erythrocyte containing ribosomes and capable of synthetizing protein [1] Based on light microscopic studies, there is evidence that the reticulocyte is in significant measure omitted in megaloblastic maturation in man [2, 3] and entirely in *opossum yolk sac megaloblastic erythropoiesis*[2] *Ribosomes* disappear and hemoglobin synthesis ceases in megaloblasts of 15-day-old C57Bl/6J mice embryos as proven by ultrastructural [4] and radioisotopic techniques [4, 5, 6] This implies that red cells derived from these megaloblasts lack ribosomes and are unable to synthesize hemoglobin *Therefore, if one accepts the presence of ribosomes and ability* to synthesize hemoglobin as characteristics of reticulocytes, then the reticulocyte stage is omitted in yolk sac megaloblastic erythropoiesis in this mouse Vitally stained megaloblasts were not evaluated in either of these studies [4, 5, 6]

*We studied yolk sac megaloblastic erythropoiesis by light micro*scopy using vitally stained fetal blood from the 11th day of intrauterine gestation up to birth of the mouse in order to determine whether or not the reticulocyte stage of maturation is omitted We chose the C57Bl/6J mouse because (1) yolk sac erythropoiesis is megaloblastic *and almost synchronous, approaching the uncomplicated kinetics in* the opossum yolk sac [7], (2) megaloblasts are easily distinguished morphologically from erythroblasts of hepatic origin, (3) cells of

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[2] BLOCK, M, unpublished observations

megaloblastic lineage of yolk sac origin containing embryonic hemoglobins [8, 9, 10, 11] resistant to acid elution may be differentiated from cells of erythroblastic lineage [12] of hepatic origin containing adult hemoglobin not resistant to acid elution

Materials and Methods

[illegible] Bar Harbor Maine [illegible]

(including yolk sac) and whole embryos were processed for sections and yolk sac and fetal blood from litter mates were used for smears

Preparation of section Fetal membranes and smaller whole embryos were fixed in Zenker formol Larger embryos were cut in half in the midsaggital plane a few minutes after fixation to insure better fixation Tissues embedded in methacrylate were sectioned at 2 μ and stained with hematoxylin-eosin azure II [13] Sections were obtained to exclude erythroblastic erythropoiesis in the yolk sac [14]

Yolk sac smears The yolk sac of 11 to 17 day embryos after separation from the embryo, other fetal membranes and placenta was streaked across a clean slide and stained with Wright's stain or with hematoxylin and erythrosin after acid elution [15, 16] as modified by Lesheaugh [12]

Pooled blood smears Twelve to 19-day-old embryos were decapitated and allowed to bleed into 4% egg albumin solution in physiologic saline This pooled blood usually from 3-4 embryos from each litter was smeared on slides both directly and after a 4 min incubation with an equal volume of 1% brilliant cresyl blue (BCB Untreated and BCB treated blood smears were then stained either with Wright's stain or by the acid elution technique [12]

BCB preparations were essential in this study for the following reasons (1) BCB treatment of blood will demonstrate cytoplasmic RNA as a reticulum network in cells not recognizable as polychromatophil in Wright stained smears (2) the cytoplasm of cells containing RNA yields a false positive stain unless the RNA is aggregated with BCB prior to acid elution [17 18] (fig 1) an important technical detail not known to American investigators

Results

Staining with Wright stain without and with pretreatment with BCB Yolk sac megaloblasts may be differentiated from hepatic erythroblasts (fig 2a vs 2b) as accurately as megaloblasts are differentiated from erythroblasts in the routine practice of hematology [19]

The first eosinophil megaloblasts were seen on day 12 when they accounted for only a minute percentage of all megaloblasts, the vast majority being polychromatophil (fig 2a) By day 15 all megaloblasts

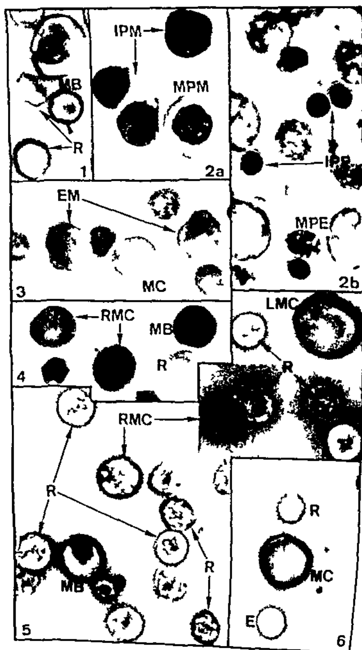
were apparently eosinophil as determined from Wright stained smears (fig. 3). When the same material was evaluated in smears stained with BCB and counterstained with Wright's, 96% of megaloblasts thought by Wright's stain to be eosinophil, contained a reticulum network (fig. 4).

The first megalocytes in Wright stained smears of yolk sac or pooled blood appeared in minute number on day 13 and until their disappearance on day 18 were always thought to be eosinophil; that is, no polychromatophilia was observed (fig. 3). Therefore, on the basis of Wright stained smears, it would appear that the reticulocyte stage was omitted. When corresponding cells were examined in slides stained by BCB and counterstained with Wright's, it became apparent that what we had considered to be eosinophil megalocytes on the basis of Wright's stain alone were BCB positive and so were reticulo-megalocytes (fig. 4). However, it was impossible to differentiate all non-nucleated red cells derived from yolk sac megaloblasts from all non-nucleated red cells derived from hepatic erythroblasts when the only distinguishing feature was size (fig. 3 and 4).

Staining with BCB followed by Betke's acid elution technique. This difficulty in differentiation of red cells of yolk sac from those of hepatic origin was resolved by use of smears stained first with BCB and thereafter by the acid elution technique. Yolk sac megaloblasts and megalocytes containing the three types of embryonic hemoglobin resistant to acid elution, were Betke positive while hepatic erythroblasts, reticulocytes and erythrocytes containing adult hemoglobin, not resistant to acid elution, were Betke negative (fig. 5).

Discussion

Yolk sac megaloblastic maturation. In our preparations 100% of megalocytes contained a BCB positive network through day 14; thereafter there was a progressive fall in percentage of BCB positive megalocytes. There are 2 possible explanations. The first and most likely is that all BCB positive megaloblasts form reticulo-megalocytes which, after about 24 h to 48 h, mature into megalocytes. An alternative explanation is that megalocytes were derived from true BCB negative megaloblasts. This however could not have occurred prior to day 15 because on days 13 and 14 all megalocytes were reticulo-megalocytes.



B T E FALVAY BLOCK S Ka ger Basel

Fig 1-6

Fig 1 Pooled blood from 15 day embryos stained by the acid elution technique without prior BCB staining to show reticulocytes (R) of probably hepatic origin (as judged by size) with false positive staining. Megaloblast from yolk sac (MB). 1,200×

Fig 2 Comparison of megaloblasts from yolk sac (2a) and erythroblasts from liver (2b) of 12 day embryo in Wright stained smears. 1,200× *a* Immature (IPM) and mature (MPM) polychromatophil megaloblasts from yolk sac. *b* Immature (IPE) and mature (MPE) polychromatophil erythroblasts from liver.

Fig 3 Cells thought to be eosinophil megaloblasts (EM) on Wright stained smear of pooled blood of 15 day embryos. Other cells presumably derived from hepatic erythroblasts except for one cell thought to be a megalocyte (MC) of yolk sac origin on basis of size. 1,200×

Fig 4 Two reticulo-megalocytes (RMC), a BCB positive megaloblast (MB) and a reticulocyte (R), the latter presumed to be of hepatic origin on basis of size, from pooled blood of 15 day embryos stained with brilliant cresyl blue and Wright's. 1,200×

Fig 5 Pooled blood from 15-day-old embryos stained with brilliant cresyl blue and then by the acid elution technique. Large Betke positive reticulo-megalocyte containing Howell Jolly body (HJMC). Betke negative hepatic reticulocytes of varied maturity with differing amounts of reticulum (R); smaller Betke positive reticulomegalocytes (RMC) which without acid elution would have been considered to be reticulocytes of hepatic origin, and a megaloblast (MB). 1,200×

Fig 6 Pooled blood of 16 day embryos stained with BCB and then by the acid elution technique. BCB negative Betke positive megalocyte (MC), a reticulocyte (R) and an erythrocyte (E), both Betke-negative, of hepatic origin. 1,200×

Correlation of structure and function of reticulocytes The presence of a BCB positive structure in megalocytes of 15 day and older embryos fulfill the light microscopic criteria for the network seen in reticulocytes which are generally thought capable of synthetizing hemoglobin Such synthetizing reticulocytes invariably contain RNA, numerous ribosomes and mitochondria [1] Reticulocytes no longer capable of hemoglobin synthesis do not incorporate tritiated leucine as shown by autoradiography, such cells contain only an occasional mitochondrion and few poly- and monoribosomes on electron microscopy [20] Yolk sac megaloblasts of 15-day-old mouse embryos contain few if any monoribosomes when embryonic hemoglobin synthesis in these cells has ceased [4], although they contain a small amount of RNA demonstrable with biochemical techniques [5] If one accepts the present concept that all reticulocytes synthetize hemoglobin, how may the demonstration of a BCB positive network in megaloblasts and megalocytes of the 15 day and older mouse embryo be explained?

In 1881 while experimenting with methylene blue, EHRLICH first recognized erythrocytes which, on staining contained an 'elegant, fine network Thus, the 'substantia reticulofilamentosa' and 'reticulocyte' have been morphologic terms based on observations made *by light microscopy* nearly 100 years ago, describing appearance and not function EHRLICH believed these cells to be degenerating erythrocytes [21]

[illegible]

Reticulocytes have been classified by HEILMEYER [22] into 4 groups according to their maturity, varying inversely with the amount of vitally stained reticulum The reticulum network observed in vitally stained megaloblasts and megalocytes of 15 day and older embryos in this study corresponds most closely to HEILMEYER's group III (faint network) and group IV cells (only a few isolated fragments of BCB positive material) We must therefore conclude that the megalocytes in 15 day and older embryos which we have shown to be reticulo-megalocytes are the progeny of the cells (megaloblasts) shown by KOVACH [4] to be incapable of synthetizing hemoglobin This conclusion is basically similar to that arrived at by MILLER and MAUNSBACH [20] in reticulocytes that *do not* synthetize hemoglobin

This study further demonstrates that eosinophilia in Wright's and presumably all Romanowsky stains, does not exclude the presence of

RNA. RNA is demonstrable with Romanowsky stains only when there are relatively large amounts in megaloblasts or megalocytes. Supravital staining with BCB or similar dye is therefore essential in order to recognize all reticulocytes.

Summary

Maturation in the overwhelming majority of megaloblasts in the C57Bl/6J mouse proceeds through a reticulo-megalocyte omitting the true eosinophil (BCB negative) megaloblast. The term 'reticulocyte' is morphologic in nature, only the more immature reticulocytes synthetize hemoglobin. We confirm that embryonic hemoglobins in megaloblasts and megalocytes of this species of mouse are resistant to acid elution, and that the BETKE KLEIHAUER technique is invalid without prior brilliant cresyl blue staining of erythroid cells.

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Bisalbuminemia of the Fast Type

Observations about a new Family

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Bis- or paralbuminemia is a familial trait characterized by the presence of an anomalous albumin with two distinct bands on electrophoresis of serum at pH 8.6. This condition, first described as 'split albumin' [12], has since been reported in about 20 families (Review by Schultze and Heremans [13], Bonazzi [1]). On the basis of the *migration rate of the anomalous albumin 2 types of paralbuminemia*, the slow and the fast, have been distinguished [17]. Later observations seem to indicate the existence of new types of paralbuminemia [3,15]. The anomaly appears to be linked to autosomal codominant alleles acting at a single locus [2, 6, 18]. In the early reports only heterozygotes have been described, while more recently some homozygous cases have been observed in which the albumin migrates as a single anomalous band [2, 3].

This paper deals with the description of an Italian family with paralbuminemia of the fast type. The anomaly has been traced over 2 generations as heterozygous condition.

Material and Methods

The propositus M. C., 68 years old, admitted to our hospital for nephrolithiasis, has been detected on routine protein study. The pedigree of his family (from Sardinia) has been traced and blood specimens have been obtained on 36 of 48 members (fig. 1).

Paper and cellulose acetate electrophoresis in barbital (pH 8.6, ionic strength 0.05) have been carried out in a Electrochimical tank using Whatman paper and Cellogel Chemetron strips. The bands have been stained in Ponceau S or Amidoschwarz and the background cleared in 5% acetic acid. The electropherogram has been analyzed with an automatic densitometer (Chromoscan, Joyce-Loebl).

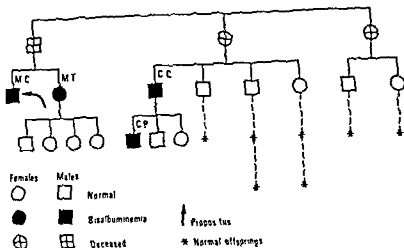


Fig 1 Pedigree of the family

elect
with
The characterization of esterase multiple forms, after separation on acrylamide support
has been achieved
acetate in 0 04 M
70 mg/100 ml The
in acid alcohol mixture

Agar gel diffusion The albumins have been separated on cellulose acetate and the

Results and Comment

The propositus, from a Sardinian family, has been fortuitously discovered while being studied for nephrolithiasis The abnormal trait has been followed for 2 generations and other 3 heterozygous cases have been detected from the 36 investigated members of the famil

em

the cases of Cooley disease have been found.

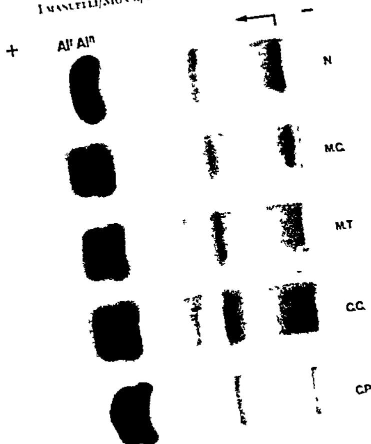


Fig. 2. Electrophoretic characterization of serum proteins on cellulose acetate support. N = normal control, M.C. = propositus

The anomalous albumin, identified in the family reported here, is of the more rare fast type. The slow and fast albumin component have been termed differently by several workers [7, 8, 9, 18]. Following WEIME's suggestion [18], however, it seems more appropriate to call Al^n the albumin with normal electrophoretic mobility, Al^f the albumin faster than normal, Al^s the albumin slower than normal. The proposed terms paralbuminemia [6] or alloalbuminemia [3] could represent the general designation for the anomalous protein.

The anomalous albumin has been separated on paper and cellulose acetate, the ratio between the two bands, Al^n and Al^f, is 1:1 (fig. 2). The electrophoretic characterization of the 2 albumin fractions has been achieved also on acrylamide support (fig. 3a). We have studied the behavior of serum esterase isoenzymes in the 4 bisalbuminemic

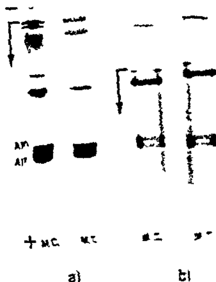


Fig. 3. Electrophoretic characteristics [illegible] on acrylamide support. [illegible]

subjects a double band [illegible] corresponding to the [illegible], has been shown (Fig. 3c). This could support the hypothesis that a[illegible] has *per se* an electrophoretic [illegible] [illegible]

The Occurrence of di[illegible] [illegible] normal human serum has [illegible] that [illegible] and [illegible] react in a manner indistinguishable from normal serum [illegible]. The [illegible] served in many of the reported cases the [illegible] relation to normal serum albumin.

Most of the families with paralbuminemia are of European [illegible] [4, 14] but an high incidence of the trait has been [illegible] American Indian tribes [3]. Weitzel [1[illegible] [illegible] where [illegible] is not determined in a family [illegible] Subsequently [illegible] families of this population [illegible] and 2 [illegible] but the relation [illegible] electrophoretic [illegible] unknown [4, [illegible]. Our [illegible] fast type [illegible] to [illegible]

Summary

Bisalbuminemia of the fast type has been observed over 2 generations in a family of Italian extraction

Data on the electrophoretic mobility and antigenic properties of the anomalous albumin are presented The characterization of esterase multiple forms shows a double band corresponding to the albumin components

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Aneuploidy in Paroxysmal Nocturnal Haemoglobinuria

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One of the most discussed haematological problems in recent years was the possible correlation between paroxysmal nocturnal haemoglobinuria (PNH) and the so-called myeloproliferative disorders The close relation of PNH and aplastic anaemia may be accepted as a fact [10] We know some adequately documented cases of PNH, in which the terminal phase was acute myeloblastic leukaemia [6, 7, 8] Dameshek [3] in a recent editorial considers PNH as a 'candidate of myeloproliferative conditions Cytogenetic studies may supply the most pregnant evidence for the relation of these pathological states The prospect of cytogenetic studies appears to be confirmed by the observation that in cases of PNH the alkaline phosphatase activity of granulocytes (GAPA) is low and this phenomenon may be indicative of the role of the chromosome pair 21 It is especially for this latter circumstance that cytogenetic studies were carried out during past years in cases of PNH In the relatively small number of investigations most workers did not observe cytogenetic deviations [1, 2 4, 5, 11] Recently Tsuchimoto *et al* [12] reported on a case, which cyto genetically appeared to be pathologic Performing direct bone marrow analysis a significant portion of the metaphases was hypoploid and the deficiency of chromosomes occurred in group C and G In most metaphases one member of the chromosome pair 21 was smaller particularly because of the deletion of the long arm of the chromosome

We are reporting on one case, which cytogenetically proved to be pathologic

The complains of the patient aged 42 years at the time of chromosome analysis started one year before He had icterus short breathing several times dark urine (haemoglobin

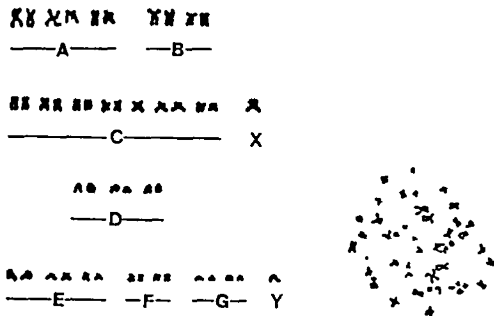


Fig 1 Metaphase containing 45 chromosomes The chromosome deficiency is in group C.

uria) The diagnosis was insured by the signs of haemolysis, pancytopenia, haemosiderinuria, positivity of the 'acid serum haemolysis' (HAM) test and the 'sucrose-haemolysis' (HARTMANN JENKINS) test, as well as by the significantly reduced GAPA Direct bone marrow analysis was performed according to the method of KIOSSOGLOU *et al* [9]

Results

The numerical result of the chromosome analysis was as follows: of 27 metaphases 11 contained 46 chromosomes, 13 – nearly 50% of cells – contained 45 and 3 contained 47 During the analysis of metaphases containing 46 chromosomes pathologic changes were not observed In the metaphases with 45 chromosomes the deficiencies were consistently observed in group C (fig 1). The analysis of the chromosome group G was carried out in 15 metaphases Although in the smaller part of metaphases studied certain differences in size were noticed, we are considering this as being a normal variation, and in this group we did not observe any anomalies Thus, in our case hypoploidy of group C was found

With the presentation of our case we wish to draw attention to the fact that the cytogenetic picture in PNH is most likely not uniform and therefore further studies are justified and perhaps also perspective.

Summary

Data of the chromosome analysis in a case of paroxysmal nocturnal haemoglobinuremia are presented. In nearly 50% of cells hypoploidy was observed in the group C. Group G showed no abberrations.

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W. A. Fuchs, J. W. Davidson and H. W. Fischer: **Lymphography in Cancer.** Recent Results in Cancer Research, vol. 23. Mit Beiträgen von G. Jantet and H. Rösler. Springer, Berlin/Heidelberg/New York 1969. IX+294 p., 189 fig., DM 76.–

Die Autoren fassen die Resultate der klinischen Lymphographie bis 1968 in einzeln bearbeiteten Kapiteln zusammen und informieren in der mit 189 Abbildungen und Skizzen gut illustrierten Monographie über die Grundlagen und diagnostischen Aussagemöglichkeiten dieser bewährten radiologischen Untersuchungsmethode. Den vorbereitenden Absätzen über die Untersuchungstechnik, die Kontrastmittel und ihre Nebenwirkungen folgt ein ausführliches und beispielhaft klares Kapitel über die normale Röntgenanatomie. Entsprechend der Zielsetzung des Werkes, eine praktische Hilfe bei der klinisch-radiologischen Diagnostik und Therapie maligner Lymphknotenveränderungen zu sein, liegt ein weiterer Schwerpunkt bei der Besprechung benigner und maligner Lymphknotenveränderungen. Dabei wird kein Zweifel über die diagnostischen und differentialdiagnostischen Schwierigkeiten der lymphographischen Interpretation gelassen. Die Aussagemöglichkeiten und ihre Grenzen werden durch die Ergebnisse vergleichender Untersuchungen zahlenmässig belegt. Eine topographisch-anatomische Grundlage für das Verständnis der Röntgenbilder vermitteln Skizzen der normalen lymphogenen Metastasierungswege verschiedener Organkarzinome. Von grosser klinischer Erfahrung zeugt das Kapitel über die malignen Lymphome, in dem eine vergleichende Deutung zwischen röntgenologischer Speicherstruktur und dem histologischen Bild versucht wird. Im letzten Teil des Buches orientieren zwei Kapitel über Indikationen, Möglichkeiten und Ergebnisse der direkten und indirekten Radioisotopen-Lymphographie und der intralymphatischen Therapie.

Das klar gegliederte Werk besticht durch seine informatorische Reichhaltigkeit bei fast durchwegs eingehaltener sachlicher Kürze. Sie wird durch gut ausgewählte Literaturhinweise ergänzt. Auch dem erfahrenen Untersucher wird diese empfehlenswerte Monographie anhand des zwölfseitigen Index ein nützlicher Ratgeber sein. Sie wendet sich vor allem an klinisch tätige Radiologen, Oncologen, Hämatologen, Chirurgen und Internisten. Für spätere Auflagen lässt sich die Prägnanz des Textes durch eine Beschneidung von Wiederholungen in den ersten, der Technik gewidmeten Kapiteln wie auch der kasuistischen Einblendungen bei den malignen Lymphomen noch verbessern. Stattdessen sollten die physiologischen Grundlagen in einem kurzen Kapitel zusammengefasst und in einem weiteren Abschnitt die systematische Beschreibung und Befundinterpretation des Lymphogramms an wenigen Beispielen erläutert werden. Die Nomenklatur der Anatomie wird sich nach dem letzten Vorschlag der ISL vereinheitlichen lassen, der dann hoffentlich für längere Zeit verbindlich bleibt. Gemessen an dem Informationsgehalt vergleichbarer Monographien ist dieses gut ausgestattete Werk mit DM 76.– als preiswürdig zu bezeichnen. Der Rezensent möchte es in seiner Handbücherei nicht missen.

M. Elke, *Basel*

Y. Najean, N. Ardaillou, C. Dresch: **Utilisation des techniques isotopiques en hématologie.** Baillère & Fils, Paris 1969.

This book is a valuable asset for the haematological literature. It is based on the experiences of the authors with radio-isotopes in haematological patients during a period of more than 10 years in the Hôpital Saint Louis (Head: Prof. Jean Bernard) in Paris. The practical use of radio-isotopes in haematology is discussed very lucidly. In the first chapter the organization and outfit of a radio-isotope laboratory in a hospital is described. Next is a chapter in which the different curves which are found when estimating the life span of blood cells in the circulation are explained; the required calculations are briefly discussed. In a third chapter the authors point out the meaning of the different pools in vivo in which administered tracer doses turn up. In a fourth chapter a general discussion follows of the

possibilities of external radiation measurements on the body surface after administration of radio-active tracers and discussion on scintigraphy. The next chapters are dealing with the different methods by which isotopes are used for diagnostic purposes in haematology. These methods are critically evaluated and causes of errors are mentioned. The description of each method is followed by a critical discussion of the interpretation of the results. Finally a chapter is devoted to therapy with radio-isotopes in haematological diseases.

It is my opinion that this book merits to be translated in other languages to make possible that a larger readerspublic may profit from its very worthwhile contents.

M. C. VERLOOP, *Utrecht*

Y. MORI und K. LENNERT: **Electron Microscopic Atlas of Lymph Node Cytology and Pathology.** Translated by K. KUCHEMANN. Springer-Verlag, Berlin/Heidelberg/New York 1969. 172 fig., XI + 309 p., DM 192,-/US-$ 52.80.

Die Zytologie – und speziell die der elektronenmikroskopisch zugänglichen Dimension



... des lymphoretikulären Gewebes, der Leukämien, der Histiozytosen und schliesslich einiger Formen von Tumormetastasen. 160 durchweg ausgezeichnete Abbildungen belegen die einleitenden Beschreibungen. Dabei erweist sich die Gegenüberstellung der lichtmikroskopischen Bilder in Semidünnschnitt-Präparaten zu elektronenmikroskopischen Vergrösserungen als besonders wertvoll. Die verarbeiteten Lymphknoten stammen von 66 Biopsien aus dem Pathologischen Institut der Universität Kiel, ergänzt durch Material, das dem Lymphknotenregister bei der Deutschen Gesellschaft für Pathologie in Kiel zugegangen war. Die didaktische Klarheit des Textes und die Qualität der Abbildungen bei erstklassiger Ausstattung des Buches durch den Verlag gestatten eine uneingeschränkte Empfehlung des Atlas für alle Morphologen, die mit den lymphatischen Geweben arbeiten.

E. GRUNDMANN, *Wuppertal*

Handbuch der Kinderheilkunde. H. OPITZ und F. SCHMID (Hrsg.). Vol. 6. Erkrankungen der Stützgewebe. Erkrankungen des Blutes und der blutbildenden Organe. Springer, Berlin/Heidelberg/New York 1967. 684 fig., XVI + 1265 p., DM 478.–

Der von H. WEICKER redigierte 6. Band des Handbuches der Kinderheilkunde behandelt in seinem ersten Teil die Pathophysiologie und Klinik der Erkrankungen von Bindegewebe, Skelett und Muskulatur. Hervorgehoben sei hier die übersichtliche Darstellung der Entwicklungsstörungen und Missbildungen, wie sie in dieser Form in den entsprechenden klassischen Lehrbüchern der Erwachsenenpathologie kaum zu finden ist.

Uns interessiert vor allem der ...

Mitwirkung von über 30 Autoren ...

Organe zur Darstellung bringt.

Grundlagen der Blutbildung, die ... und der Stoffwechsel der einzelnen Zell-

systeme und die hämatologischen Laboratoriumsmethoden behandelt Von speziellem Interesse sind die Kapitel über die Besonderheiten der pädiatrischen Hämatologie und über die Normalwerte und ihre Wandlungen im Laufe der kindlichen Entwicklung während andere Abschnitte sich in ihrer Aussage nicht von den entsprechenden Darstellungen der Erwachsenen Literatur unterscheiden Die folgenden Teile behandeln die Pathologie und Klinik des *erythrozytären Systems, des leukozytären Systems* und der *hämorrhagischen Diathesen* Die Anämien werden nach ihrer Pathogenese gegliedert in Differenzierungsstörungen (aplastische und hypoplastische Anämien), Teilungsstörungen (megaloblastäre Anämien, Erythroblastopenien), Störungen der Hämoglobinsynthese, pseudoaplastische Anämien, Blutungsanämien und hämolytische Anämien Beim Studium dieser Abschnitte tritt die ganze Problematik der Klassierung der Anämien hervor So werden z B die Erythroblastopenien zusammen mit den megaloblastären Anämien besprochen und damit aus dem Rahmen der hypoplastischen bzw aplastischen Anämien herausgenommen Die Eisenmangelanämien werden im Kapitel der Hämoglobinopathien aufgeführt Hier finden wir auch die Thalassämien bei den Anomalien der Hämoglobinstruktur, nicht aber im Abschnitt der gestörten Hämoglobinsynthese (sideroachrestische Anämien, Bleianämie usw) Die Krankheitsbilder mit erhöhter Erythrozytenzahl beschliessen die Pathologie der Erythropoese Neben der sehr seltenen Polycythaemia vera wird hier die Pathogenese der *Polyglobulien klar und ausführlich dargestellt Das Kapitel der leukozytären* Erkrankungen enthält neben den hereditären Anomalien den reaktiven Veränderungen, den Neutropenien (inkl Agranulozytose) und den Leukämien und Retikulosen auch die Erkrankungen von Lymphknoten, Thymus und Milz Neben den kindlichen Leukämien sind es vor allem die Retikulosen deren Darstellung durch den Pädiater unsere Kenntnisse wesentlich bereichert Die hämorrhagischen Diathesen beschliessen das Werk Sie werden in der üblichen Klassierung in vaskuläre, thrombozytogene und plasmatische Störungen besprochen, wobei auch hier wieder die Besonderheiten der kindlichen Pathologie und die hereditären Anomalien hervorzuheben sind

Trotz der grossen Zahl von Autoren liegt hier ein einheitliches Werk über die pädiatrische Hämatologie vor uns, das neben seinem Handbuchcharakter die einzelnen hämatologischen Erkrankungen übersichtlich, in didaktisch vorzüglichem Aufbau und durch sehr instruktive zum Teil farbige Abbildungen illustriert wiedergibt Die jedem Kapitel angeschlossenen Literaturverzeichnisse berücksichtigen die neueren Arbeiten bis zum Jahre 1966

H Lüdin *Basel*

Varia

World Federation of Haemophilia

The VIIth Congress of the World Federation of Haemophilia together with a Symposium on Blood Transfusion and Blood Utilization is to be held in Tehran from May 17 to 20 1971

For further information Dr F Ala Secretary General VIIth Congress of the World Federation of Haemophilia PO Box 13 1438 CICJ *Tehran* (Iran

Acta haemat 44 257 264 (1970)

Surface Ultramicroscopy of Paroxysmal Nocturnal Haemoglobinuria Erythrocytes

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Institute of Medical Pathology University of Milan Milan

Paroxysmal nocturnal hemoglobinuria (PNH) is a hemolytic disorder characterized by an acquired abnormality of the erythrocytes which renders them sensitive to complement lysis [17, 19] PNH erythrocytes have been studied quite extensively under the electron microscope and the results have been conflicting In fact, according to some authors [2 3, 15] PNH red cells have an unusually pitted and patchy surface, while to others [9, 10, 14] PNH red cell membrane does not appear to be different from normal This variability of results might depend on the fact that the red cell membrane preparation by drastic osmotic hemolysis probably results in damage of the membranous structure The technique described by DANON *et al* [6] obviates this disadvantage in that lysis is gradual Using this method, LEWIS *et al* [13] found that PNH red cell membrane shows surface pitting and an abnormal electron dense material The authors conclude that these findings may be an artifact induced *in vitro* during preparation for electron microscope, but associated with the basic structural defect of PNH red cells

Recently the scanning electron microscope (SEM) has been introduced in hematology to study the appearance of blood cells and particularly of their surface [4 5, 8, 18] As far as we know, PNH erythrocytes have been examined with this three dimensional technique only by SALSBURY and CLARKE [18] who observed an extraordinary plasticity of the red cells in this disorder

The purpose of this paper is to report the results of our study of PNH erythrocytes by scanning electron microscopy

Materials and Methods

Samples of venous blood were drawn with acid-citrate-dextrose from 4 patients affected by PNH (table I) and 4 normal healthy adults. The PNH *patients had not received blood transfusions in the previous few weeks.*

The blood was centrifuged at 1,500 *g* for 10 min and the supernatant removed. The red cells were washed 3 times with saline and *then fixed in 0.5% phosphate-buffer* glutaraldehyde (pH 7.4) for 1 h at room temperature [12]. After a triple rinse in distilled water for 30 min the erythrocytes were resuspended in distilled water. One drop of this suspension was placed and dried on a supported disk. After coating with gold platinum in a Hitachi vacuum chamber, the specimens were examined in a Cambridge Stereoscan electron microscope at an angle of 45° using Tri-X Kodak film.

Results

The erythrocytes of the 4 examined patients showed some aspects that were not observed in the control subjects (fig. 1–11).

1. They varied considerably in size and shape. In addition to the normally-shaped biconcave cells, erythrocytes with a less pronounced central depression and/or with a crenated peripheric rim were frequently observed.

2. Most of them resembled the erythrocytes seen numerous by SALSBURY and CLARKE [18] in thalassemia major and considered to be target cells; they *showed a roundish, large protuberance raising* from the middle of the depression. Actually the same type of cells

Table I. Some clinical and hematological data of patients at time of study

Patient	Age in years	Sex	Hb, g/100 ml	Reticulocytes, %	Acidified serum lysis, %	Acetylcholinesterase inhibition, %	Comment
C.G.	37	F	5.2	0.4	6	32	Aplastic
P.L.	42	F	8.1	8.0	30	73	Splenectomized
D.I.	43	F	12.6	0.6	5	77	Previously aplastic recovered after androgen treatment
B.A.	22	M	10.2	2.0	20	52	







Fig 1 Variability in shape of PNH erythrocytes One erythrocyte shows a crenated peripheric rim and a central protuberance (patient D L., × 3,800)

Fig 2 PNH erythrocyte with a crenated peripheric rim and a central depression less marked than normal (patient B A , × 8,800)

Fig 3 PNH erythrocyte with a marked irregular shape, pits and deep craters are visible on the surface (patient P L., × 8 800)






Fig. 4. PNH erythrocyte with a protuberance raising from the internal rim (patient D.I. × 8,800).

Fig. 5. PNH erythrocyte. The biconcave shape is lost, the peripheral rim is crenated, two protuberances are evident in the center (patient B.A. × 8,800).

Fig. 6 and *7.* PNH erythrocytes with a protuberance raising from the central depression (patients P.I. and C.C. × 8,800).



Fig. 8 PNH erythrocytes with a protuberance raising from the central depression (patients P I and C G × 8 800

Fig. 9 PNH erythrocyte with two central protuberances and small superficial depressions on the surface (patient C G × 8 800)

Fig. 10 PNH erythrocyte with a protuberance raising from the internal rim and oriented towards the central hollow patient D I × 7 800)

Fig. 11 PNH erythrocyte with a protuberance raising from the internal rim and oriented towards the central hollow patient P L × 15 500)









Fig. 4. PNH erythrocyte with a protuberance raising from the internal rim (patient D.I.). × 8,800.

Fig. 5. PNH erythrocyte. The biconcave shape is lost; the peripherical rim is crenated; two protuberances are evident in the center (patient B.A.). × 8,800.

Fig. 6 and *7.* PNH erythrocytes with a protuberance raising from the central depression (patients P.I. and C.C.). × 8,800.









Fig 8 PNH erythrocytes with a protuberance raising from the central depression (patients P L and C G × 8 800)

Fig 9 PNH erythrocyte with two central protuberances and small superficial depressions on the surface (patient C G × 8 800

Fig 10 PNH erythrocyte with a protuberance raising from the internal rim and oriented towards the central hollow (patient D I × 7 800

Fig 11 PNH erythrocyte with a protuberance raising from the internal rim and oriented towards the central hollow (patient P L., × 15 500

was observed in normal subjects too, although rarely. Occasionally in PNH cells there were two protuberances and sometimes they protruded from the internal rim of the erythrocyte towards the central hollow.

3. The surface of some cells showed deep craters, pits and small superficial depressions. These features were found almost exclusively in the more irregular red cells, i.e. those which had lost their biconcave shape.

No relationship appeared to exist between the above mentioned findings and the severity of the disease, the degree of lysis in the acidified serum test and the decrease of erythrocyte acetylcholinesterase activity.

Discussion

The marked variability in size and shape of PNH erythrocytes might depend on the presence of red cells of different age, which is a common finding in hemolytic anemias.

Different kinds of protuberances have been described in various conditions: newborns [16], splenectomized subjects [16], patients with autoimmune hemolytic anemia [18] or acanthocytosis [11].

Moreover a central protuberance is said to be a feature of target cells [18]. Their presence in the PNH samples examined was unexpected, since no target cells were observed with the optical microscope in the blood smears of the same patients. The significance of the different types of protuberances observed in PNH cannot be easily explained. It has been shown by BESSIS *et al.* [1] and by KAYDEN and BESSIS [11], that protuberances can occur in the red cell as a consequence of washing in saline after glutaraldehyde fixation: they disappear if the red cell is normal but persist in the case of acanthocytosis and sickle cell anemia. Following BESSIS *et al.* [1] therefore the protuberances observed in PNH should not be a consequence of washing but possibly of an abnormality of the red cell. Protuberances from the rim of the red cells in newborn, and splenectomized subjects have been interpreted by PRESTON and SHAHANI [16] as an expression of extrusion of solid particles from within the cell, possibly due to the absence of a normal splenic function. In PNH cells inclusions could represent the residual material shown by LEWIS *et al.* [13] with the electron microscope.

Pits in PNH red cells have been observed with the electron microscope [2, 3, 13, 15] LEWIS *et al* [13] concluded that this finding may be indicative of a structural defect of the red cell surface which predisposes the cell to pitting during preparation of the membrane for electron microscopy Pits, craters and depressions, observed with the SEM almost exclusively on the more irregular red cells, support this possibility, since they might develop as a consequence of crumpling of the surface of the more altered red cells during their manipulation The low frequency of these morphologic abnormalities in our hands, as compared to that found by others [2, 3, 13, 15] with the transmission electron microscope, is probably to be explained with the lower resolution power of SEM It is to be noted that pits and craters similar to those seen in PNH have been observed with SEM in newborns [16], splenectomized subjects [16] patients suffering from some kind of hemolytic anemias [18] or sideroblastic anemias [18]

In conclusion, the present investigation has not shown any morphologic abnormality specific for PNH red cells Moreover, SEM allows only detection of gross alterations and it is still not clear how many of these are due to artifacts created during the specimen preparation However, since these morphologic abnormalities as a rule are not found in normals, they can be considered as an expression of the basic structural defect of PNH red cell membrane

Summary

The morphology of erythrocytes from 4 patients with paroxysmal nocturnal hemoglobinuria (PNH) was studied by scanning electron microscope (SEM) A great variability in shape and size of red cells was observed many red cells showed protuberances raising from the central depression or from [illegible]
pits craters and depressions were [illegible]
PNH and the creation of artifacts [illegible]
they can be an expression of the basic structural defect of PNH red cell membrane

Acknowledgement

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Lysosomale Enzyme in Lymphozyten

II Chronische lymphatische Leukämie (CLL): Veränderungen des Enzymgehaltes (saure Phosphatase β Glucuronidase) von Blutlymphozyten während einer mehrtägigen Stimulierung mit Phytohämagglutinin *in vitro*[1]

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Im Vergleich zu Granulozyten enthalten Blutlymphozyten gesunder Menschen nur wenige Lysosomen und niedrige Aktivitäten lysosomaler Enzyme [1, 2, 9 11] Werden diese Zellen jedoch *in vitro* mit Phytohämagglutinin (PHA) inkubiert, so sind bereits wenige Stunden nach Versuchsbeginn eine Zunahme der Lysosomenzahl und ein Anstieg der Aktivität lysosomaler Enzyme zu beobachten [8, 15, 18] Diese Veränderungen nehmen bis zum 3 Stimulierungstag dem Zeitpunkt der maximalen mitotischen Aktivität der Zellen, zu [1, 8, 18] Da sich die Lysosomenzahl kurz vor der Mitose wieder verringert, wird die Möglichkeit diskutiert dass lysosomale Enzyme in Beziehung zu Stoffwechselprozessen stehen, die die Mitose auslösen und/oder begleiten [1, 10, 11] Blutlymphozyten von Patienten mit chronischer lymphatischer Leukämie (CLL) zeigen eine verminderte Reaktion auf PHA [7] die ausserdem verzögert, d h erst nach 5 bis 7 Tagen eintritt [6] Im Vergleich zu normalen unstimulierten Lymphozyten ist in diesen Zellen die Aktivität der lysosomalen Enzyme saure Phosphatase und β Glucuronidase häufig stark vermindert [3 4] Stimulierte CLL Lymphozyten («CLL-Blasten») unterscheiden sich weder licht noch elektronenmikroskopisch [5] wesentlich von PHA Blasten, die durch Transformation normaler Lymphozyten entstanden sind

In den hier mitgeteilten zyto- und biochemischen Untersuchungen wurde der Frage nachgegangen ob in CLL Lymphozyten während

Herrn Prof Dr O H Arnold zum 60 Geburtstag in Dankbarkeit gewidmet

[1] Auszugsweise vorgetragen bei der 76 Tagung der Deutschen Gesellschaft für Innere Medizin (Wiesbaden 6 9 Apr 1970)

einer mehrtägigen Inkubation mit PHA Lysosomenveränderungen nachgewiesen werden konnen, wie sie in stimulierten normalen Lymphozyten zu beobachten sind.

Material und Methoden

Patienten

Die Untersuchungen wurden an 3 gesunden Blutspendern und 7 Patienten mit CLL durchgeführt. Die Blutlymphozytenzahlen der CLL-Patienten und die Therapie sind in Tabelle I angegeben.

Zellgewinnung, Kulturverfahren, präparative Massnahmen und biochemische Analysen

Die für die Versuche erforderlichen reinen Lymphozyten-Suspensionen wurden nach einem früher beschriebenen Verfahren aus heparinisiertem Frischblut gewonnen [2]. Die für die biochemischen Studien vorgesehenen Kulturen (Einzelheiten des Kulturverfahrens bei [4]) enthielten $0,91 \pm 0,12 \times 10^6$ Zellen/ml Kulturmedium (Gesamtvolumen 40 ml). Der Zellgehalt der Kulturen, die zur Bestimmung der Transformationsrate, zum zytochemischen Nachweis der sauren Phosphatase und zur Messung des ^{3}H-Thymidin Einbaus dienten, betrug $0,75 \times 10^6$/ml (Gesamtvolumen 4 ml).

Die Stimulierung erfolgte mit Phytohämagglutinin (PHA P, Difco Lab., Detroit, Mich., USA). Der Inhalt eines Fläschchens wurde *in 2,5 ml Aqua bidest. gelöst. Davon wurden* 0,02 ml/ml Medium zugesetzt, da sich durch Vortestung gezeigt hatte, dass mit dieser PHA-Konzentration eine optimale Blastentransformation zu erzielen war. Als Kontrollen dienten Lymphozyten Suspensionen mit Zusatz von 0,9%iger NaCl Lösung (0,02 ml/ml Medium).

Zur Beurteilung der Blastentransformation wurden die Kulturen nach der von Hirschhorn [12] mitgeteilten Methode (ohne vorherigen Zusatz von Vincaleukoblastin) mit einem Alkohol Essigsäure Gemisch fixiert und mit saurem Orcein (0,5%) gefärbt. Die Auswertung fand im Phasenkontrast-Mikroskop statt. Die zytochemische Bestimmung der sauren Phosphatase erfolgte nach der von Hirschhorn *et al.* [11] *verwendeten modifizierten* Gomori-Methode. Die Zellen wurden in Serum vom fötalen Kalb resuspendiert, während 1¾ h bei 4°C in 10%igem, azetat (0,1 M) – gepufferten Formalin fixiert und anschliessend 4 h mit dem Substrat (D, L-β Glycerophosphat, Dinatriumsalz, Alpha Isomer-Gehalt unter

Tabelle I. CLL-Patienten. Blutlymphozytenzahlen und Therapie

Name	Alter (Jahre)	Geschlecht	Lymphozytenzahl pro µl	Therapie
W F	66	♀	8000	O
L. M	67	♂	31000	O
W K	56	♀	41360	O
K E.	54	♂	93600	Chlorambucil und Prednison
Sch H	76	♂	106232	Chlorambucil und Prednison
R P	47	♂	103750	O
St R	54	♂	197160	O

0,1%, Sigma Chem Co, St Louis, Mo, USA) inkubiert Bei der Auswertung wurde der Prozentsatz der Zellen bestimmt, die mehr als 3 Granula des braunschwarzen Reaktionsproduktes enthielten

Als Mass für die DNS-Synthese diente der ^{3}H Thymidin Einbau in die Lymphozyten [illegible]

[illegible]

in einem Szintillationszähler (Liquid Scintillation Spectrometer Tri-Carb, Model 3003, [illegible]

[illegible] erforderlich

Die für die biochemischen Untersuchungen vorgesehenen Kulturen wurden nach einem früher beschriebenen Verfahren [4] aufbereitet Die Aktivität der sauren Phosphatase, β-Glucuronidase und Malat Dehydrogenase (MDH) sowie der Proteingehalt wurden mit früher beschriebenen Methoden [2] bestimmt.

Berechnungen

Für die Berechnung der Signifikanz von Unterschieden wurde der t Test nach Student verwendet Es werden die Mittelwerte ($\bar{x}$) und die Standardabweichungen der Mittelwerte ($s_{\bar{x}}$) angegeben

Ergebnisse

In den gereinigten Lymphozyten-Suspensionen von Patienten mit CLL fanden sich 100%, von Normalpersonen 98–99% mononukleäre Zellen Zu Versuchsbeginn wurden in allen Lymphozyten-Suspensionen 97–100% vitale Zellen nachgewiesen Die während einer 7tägigen Stimulierung von normalen und CLL-Lymphozyten gemessenen Aktivitäten der sauren Phosphatase (biochemische Bestimmung), β Glucuronidase und MDH sowie der Proteingehalt, die Transformationsrate, die DNS-Synthese (^{3}H-Thymidin-Einbau) und die Aktivität der zytochemisch nachweisbaren sauren Phosphatase dieser Zellen sind in Tabelle II und Abbildung 1 dargestellt. Die Ergebnisse der biochemischen Untersuchungen wurden auf die zu Versuchsbeginn pro Kultur eingesetzte Zellzahl bezogen. Während unstimulierte normale Lymphozyten eine Transformation vermissen liessen und keine nennenswerte Aktivität der zytochemisch nachweisbaren sauren Phosphatase zeigten, waren nach 3tägiger Inkubation mit PHA im Mittel 53,6% der Zellen transformiert und 31% deutlich bis stark saure Phosphatase-positiv (Abb 2a) Nach einem leichten Anstieg am 5. Tag ging die mittlere Transformationsrate am 7. Tag auf Werte

Tabelle II. Biochemische und morphologische Untersuchungen an Lymphozyten von Inkubation mit PHA und 0,9%iger

	Versuchsbeginn		3. Tag
	Kontrolle	PHA	Kontrolle
Normale Lymphozyten			
DNS-Synthese (^{3}H-Thymidin-Einbau, cpm × 10^{-3})	0,44 ± 0,04	0,57 ± 0,08	2,67 ± 0,39
Transformationsrate (% Blasten)	0,0	0,0	0,8 ± 0,6
Saure Phosphatase (zytochem. Bestimmung, % pos. Zellen)	0,0	0,0	1,3 ± 0,9
Saure Phosphatase (pro 10^7 Zellen, biochem. Bestimmung)	153,3 ± 11,6		94,8 ± 5,8
β-Glucuronidase (pro 10^7 Zellen)	50,6 ± 6,9		35,0 ± 3,1
Malat-Dehydrogenase (pro 10^7 Zellen)	303,6 ± 43,3		199,7 ± 35,8
Protein (pro 10^7 Zellen)	139,4 ± 14,6		82,6 ± 17,2
CLL-Lymphozyten			
DNS-Synthese (^{3}H-Thymidin-Einbau, cpm × 10^{-3})	0,39 ± 0,05	0,41 ± 0,06	0,61 ± 0,08
Transformationsrate (% Blasten)	0,0	0,0	0,2 ± 0,1
Saure Phosphatase (zytochem. Bestimmung, % pos. Zellen)	0,0	0,0	0,0
Saure Phosphatase (pro 10^7 Zellen, biochem. Bestimmung)	63,8 ± 17,44		36,2 ± 7,6
β-Glucuronidase (pro 10^7 Zellen)	18,1 ± 5,0		11,1 ± 2,7
Malat-Dehydrogenase (pro 10^7 Zellen)	262,9 ± 52,3		102,2 ± 19,3
Protein (pro 10^7 Zellen)	157,6 ± 41,6		216,0 ± 85,2

Aktivitätseinheiten: Saure Phosphatase: mμMol freiges Pi/h. β-Glucuronidase: mμMol freiges Phenolphthalein/h. Malat-Dehydrogenase (MDH): mμMol oxyd. NADH/min

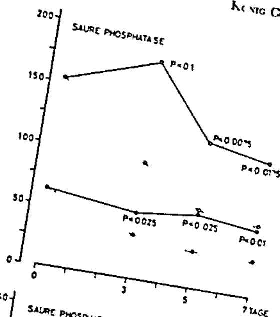
unter 50% zurück. Die Aktivität der zytochemisch nachweisbaren sauren Phosphatase war am 5. Tag auf 17% und am 7. Tag auf 10,7% abgefallen. In Kulturen von CLL-Lymphozyten trat eine nennenswerte Transformation erst nach 5 bis 7 Tagen ein, ohne dass der bei normalen Lymphozyten nachgewiesene hohe Stimulierungsgrad erreicht wurde. Wie aus Tabelle II hervorgeht, war in den stimulierten CLL-Lymphozyten eine gesteigerte DNS-Synthese festzustellen, die

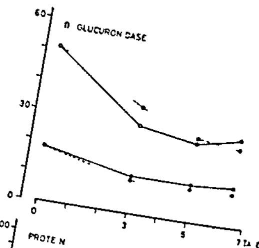
Gesunden (n = 3) und Patienten mit CLL (n = 7) vor und nach 3 5- und 7tägiger NaCl Losung (Kontrollen)

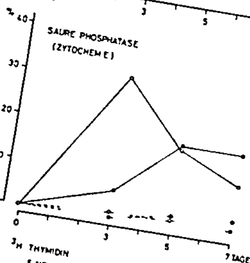
	5 Tag		7 Tag	
PHA	Kontrolle	PHA	Kontrolle	PHA
39 00 ± 2 55	4 69 ± 0 65	11 81 ± 1 8	3 64 ± 0 91	10 26 ± 2 30
36 ± 8 6	1 4 ± 0 7	58 1 ± 15 4	0 6 ± 0 8	47 4 ± 15 6
31 0 ± 6 4	1,5 ± 0 5	17 0 ± 5 5	3 8 ± 1 3	10 7 ± 3 5
177 8 ± 51 8	64 8 ± 6 2	120 5 ± 7 4	56 3 ± 10 0	112 8 ± 12 4
29 2 ± 3 8	28 0 ± 9 9	26 1 ± 4 8	26 7 ± 1[illegible] 0	29 8 ± 5 0
727 9 ± 225 8	131 7 ± 37 2	541 8 ± 1 4 4	110 5 ± 23 6	335 6 ± 90 2
373 7 ± 153 1	99 7 ± 24,5	174 9 ± 22 4	68 1 ± 14 1	124 4 ± 45 8
3 90 ± 1 00	1 34 ± 0 25	8 66 ± 2 25	1 37 ± 0 30	8 37 ± 1 76
2 4 ± 0 8	0 5 ± 0 5	22 4 ± 9 2	0 2 ± 0 1	20 7 ± 9 1
6 0 ± 1 9	2 3 ± 1 3	18 0 ± 6 8	0 8 ± 0 6	18 2 ± 8 1
53 8 ± 12 2	31 1 ± 8 7	60 1 ± 17 4	31 7 ± 13 4	56 3 ± 16 9
11 9 ± 3 7	11,2 ± 4 6	12 3 ± 3 8	11 2 ± 4,8	12 9 ± 5 2
139 4 ± 41 2	64 0 ± 15 1	249 2 ± 105 0	124 7 ± 48 3	4 7 7 ± 278 0
288 9 ± 95 0	146 0 ± 59 5	224 0 ± 56 4	151 9 ± 65 7	40[illegible] 4 ± 168 4

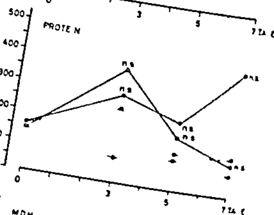
Protein mg 10 Zellen

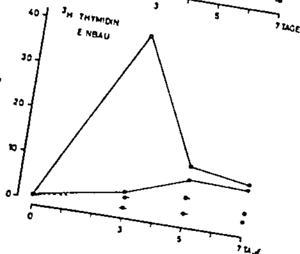
wie die Transformationsrate ihr Maximum am 5 und 7 Tag erreichte Diesen Veranderungen ging eine Erholung der Aktivitat der zytochemisch nachweisbaren sauren Phosphatase parallel Der Prozentsatz positiver Zellen (etwa 18%) und die Intensitat der Reaktion waren jedoch geringer als in maximal stimulierten Kulturen normaler Lymphozyten (Abb 2b) Bei biochemischer Untersuchung zeigten unstimulierte Kulturen normaler Lymphozyten wahrend der Versuchs


SAURE PHOSPHATASE
200
150
100
50
0
P<0.1
P<0.0025
P<0.0125
P<0.025
P<0.025
P<0.01
0
3
5
7 TAGE
NORMALE LYMPHOZYTEN PHA
NORMALE LYMPHOZYTEN KONTROLLE
CLL LYMPHOZYTEN PHA
CLL LYMPHOZYTEN KONTROLLE


60
30
0
0
3
5


%
40
SAURE PHOSPHATASE
30
20
10
0
0
3
5
7 TAGE


500
PROTEIN
400
300
200
0
n.s.
n.s.
n.s.
n.s.
0
3
5


40
30
20
10
0
0
3
5

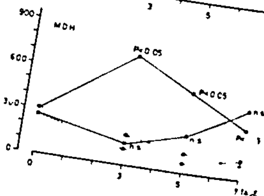

900
MDH
600
0
P<0.05
P<0.05
n.s.
n.s.
0
3
5

Abb. 1

zeit eine kontinuierliche Verminderung der Aktivitaten aller untersuchten Enzyme und des Proteingehaltes Nach Stimulierung mit PHA stiegen die Aktivität der sauren Phosphatase nur gering, die Aktivitat der MDH und der Proteingehalt deutlich an Die aus diesem unterschiedlichen Verhalten der unstimulierten und stimulierten Zellen resultierende Differenz war 3 Tage nach Stimulierungsbeginn am starksten ausgepragt, wurde jedoch für die saure Phosphatase erst nach 5 und 7 Tagen signifikant Die Aktivität der β-Glucuronidase verminderte sich wahrend der Versuchszeit in unstimulierten und stimulierten Kulturen, ohne dass sich jedoch signifikante Differenzen zwischen PHA-behandelten und unbehandelten Zellen nachweisen liessen.

In Kulturen unstimulierter CLL-Lymphozyten war wie in entsprechenden Ansatzen normaler Zellen ein Abfall der Enzymaktivitäten nachweisbar Der Proteingehalt stieg am 3 Tag an und sank am 5 Tag wieder auf den Ausgangswert ab In PHA stimulierten CLL-



Abb 2 Zytochemische Darstellung der Aktivität saurer Phosphatase (modifizierte Gomori Methode) in transform erten Lym [illegible]
(a) n [illegible]
PHA [illegible]
korn [illegible]

Abb 1 Aktivität von saurer Phosphatase (biochem [illegible]

Tabelle III Spezifische Aktivitäten von saurer Phosphatase, β Glucuronidase und Malat mit CLI (n = 7) vor und nach 3-, 5- und 7tägiger

Normalpersonen	Saure Phosphatase Versuchsbeginn 1 29±0 23				β-Glucuronidase 35 3±7 5	
	Kontrolle		PHA		Kontrolle	
3 Tag	1 14±0 20	n s	1 39±0 87	n s	40 2±10 5	n s
5 Tag	0 69±0 06	n s	0 70±0 05	n s	29 4± 9 0	n s
7 Tag	0 83±0 01	n s	1 19±0 38	n s	30 0± 9 0	n s
CLI	Versuchsbeginn 0 42±0 06				12 3±2 2	
	Kontrolle		PHA		Kontrolle	
3 Tag	0 31±0 11	n s	0 31±0 13	n s	9 8± 4 5	n s
5 Tag	0 57±0 20	n s	0 30±0 09	n s	21 3±10 4	n s
7 Tag	0 35±0 14	n s	0 17±0 04	P<0 005	15 1± 7 7	n s

Aktivitätseinheiten Saure Phosphatase μMol freies P/h mg Protein β-Glucuronidase μMol freies Phenolphthalein 1/100 mg Protein Malat Dehydrogenase (MDH) μMol oxyd NADH$_2$/h mg Protein

Lymphozyten lag die Aktivität der sauren Phosphatase signifikant höher als in unstimulierten Zellen Dieser Unterschied erreichte sein Maximum erst am 5 Tag nach PHA-Zusatz Vom 3 bzw 5 Versuchstag an waren der Proteingehalt und die Aktivität der MDH in PHA-behandelten Lymphozyten gegenüber den Kontrollansätzen nicht signifikant erhöht Unterschiede im Verhalten der β Glucuronidase Aktivität waren zwischen unstimulierten und stimulierten CLL-Lymphozyten und entsprechenden normalen Zellen nicht nachzuweisen

Tabelle III gibt die spezifischen Enzymaktivitäten wieder, die in normalen und CLL-Lymphozyten während der mehrtägigen Versuchsperiode bestimmt wurden Sie blieben bei normalen und CLL-Lymphozyten gegenüber den Ausgangswerten unverändert oder sanken signifikant ab In PHA stimulierten normalen Lymphozyten fiel ein Anstieg der spezifischen Aktivität der sauren Phosphatase und MDH auf, diese Veränderungen liessen sich jedoch nicht statistisch sichern

Dehydrogenase (MDH) in Lymphozyten von Normalpersonen (n = 3) und Patienten Inkubation mit PHA und 0,9%iger NaCl Lösung (Kontrollen)

		Malat Dehydrogenase 127,3 ± 16,4			
PHA		Kontrolle		PHA	
15,6 ± 3,2	P < 0,05	130,0 ± 30,4	n.s.	193,5 ± 33,0	n.s.
14,8 ± 1,6	P < 0,025	109,4 ± 37,8	n.s.	176,1 ± 38,3	n.s.
28,6 ± 6,3	n.s.	98,2 ± 13,5	n.s.	174,2 ± 24,5	n.s.
		116,3 ± 44,9			
PHA		Kontrolle		PHA	
6,6 ± 2,1	P < 0,05	44,9 ± 13,6	P < 0,01	49,2 ± 16,9	P < 0,025
7,4 ± 2,2	n.s.	48,9 ± 17,2	P < 0,025	70,1 ± 20,9	n.s.
6,2 ± 2,9	n.s.	65,3 ± 23,0	n.s.	65,1 ± 12,6	P < 0,05

t Test Ausgangswerte gegen Werte am 3, 5 und 7 Versuchstag n.s. nicht signifikant

Diskussion

Die während der 7tägigen Versuchsperiode in Kulturen unstimulierter normaler Lymphozyten beobachtete Abnahme der Aktivität aller untersuchten Enzyme und des Proteingehaltes ist als Folge einer Verminderung der Zellzahl anzusehen, wie sie von verschiedenen Autoren [19, 24, 25] direkt gemessen wurde. Darauf weisen auch die spezifischen Enzymaktivitäten hin, die keine signifikanten Abweichungen vom Ausgangswert erkennen liessen.

Im Gegensatz dazu stieg die Aktivität der sauren Phosphatase in Kulturen PHA stimulierter Zellen gegenuber dem Ausgangswert leicht, die der MDH deutlich an, wenn die Enzymaktivitäten auf die zu Versuchsbeginn eingesetzten Zellzahlen bezogen wurden. Diese Befunde sprechen für eine Neusynthese und/oder Aktivierung beider Enzyme in den transformierten Zellen, beweisen diese Annahme jedoch nicht endgultig, da nach Stimulierungsbeginn wegen der starken PHA bedingten Lymphozytenagglutination keine Zellzählungen erfolgten und somit eine während der Versuchsperiode ein

getretene Erhöhung der Zellzahlen mit entsprechender Zunahme der Enzymaktivitäten nicht ausgeschlossen wurde.

Verschiedene Autoren [19, 24, 25, 26] bestimmten die Zahl der in PHA-behandelten und in Kontroll-Kulturen nach mehreren Tagen vorhandenen Lymphozyten bzw. Lymphozytenkerne, nachdem die in PHA-Kulturen gebildeten Agglutinate durch Zusatz verschiedener Agentien weitgehend zum Verschwinden gebracht worden waren. Schellekens und Eijsvoogel [24] stellten fest, dass die Zellzahl am 3. Tag in unstimulierten Kulturen um etwa 14% und in PHA-Kulturen um etwa 11% geringer war als zu Versuchsbeginn. Yam *et al.* [26] finden zum gleichen Zeitpunkt in PHA-Kulturen einen Abfall um etwa 40% des Ausgangswertes. Im Gegensatz dazu beschrieben Rabinowitz *et al.* [19] sowie Stewart und Ingram [25], dass in PHA-Kulturen nach 3 Tagen entweder die Ausgangszellzahl wieder erreicht oder sogar überschritten wurde, während die Zellen in Kontrollkulturen deutlich vermindert waren. Die Ergebnisse der in PHA behandelten Kulturen durchgeführten Zellzählungen sind wegen der unterschiedlichen Methodik nicht miteinander vergleichbar und gestatten keine endgültige Aussage über die Veränderungen der Lymphozytenzahl unter diesen Versuchsbedingungen.

Die Tatsache, dass die Aktivität der β-Glucuronidase in Kulturen stimulierter normaler Lymphozyten wie in Kontrollkulturen deutlich abnahm, spricht dafür, dass auch nach PHA-Zusatz die Zellzahl zurückging. Setzt man eine derartige Zellverminderung voraus, so kann der Anstieg der Aktivität der sauren Phosphatase und der MDH über den Ausgangswert nur durch eine Neusynthese und/oder Aktivierung dieser Enzyme in den verbliebenen Zellen erklärt werden. Die in einem Teil der Experimente nachgewiesene Erniedrigung der spezifischen Enzymaktivitäten war durch den während der Versuchszeit beobachteten erheblichen Anstieg des Proteingehaltes in den PHA-Kulturen bedingt. Es ist möglich, dass ein Teil dieses Proteins nicht in den Lymphozyten neu synthetisiert wurde, sondern aus dem Kulturmedium stammte und von den zur verstärkten Endozytose [13, 20] befähigten Lymphozyten inkorporiert wurde oder an der Zelloberfläche fixiert war.

Dass die Aktivität der sauren Phosphatase in normalen Lymphozyten nach 3tägiger Stimulierung mit PHA höher war als in unstimulierten Kontrollzellen, wurde auch von Hirschhorn *et al.* [8, 11] beobachtet. Dagegen wurde zu diesem Zeitpunkt eine Änderung der

β Glucuronidase-Aktivität vermisst, ein Befund, der durch unsere Resultate bestätigt wird NADLER *et al* [16] sahen in den Kontrollkulturen während der ersten 3 Tage einen kontinuierlichen Abfall der spezifischen Aktivität der sauren Phosphatase, während in den PHA-Kulturen nach einer initialen Verminderung ein Anstieg mit maximalen Werten zwischen dem 2 und 3 Stimulierungstag auffiel Anschliessend sank die Aktivität erneut steil ab, um 3 Tage nach Stimulierungsbeginn annähernd den Wert der Kontrollkulturen zu erreichen Wurde die Enzymaktivität in PHA-Kulturen auf die eingesetzte Zellzahl und den Ausgangswert bezogen, so war zwischen der 6 und 60 Stunde eine Erhöhung und nach 72 Stunden eine Erniedrigung zu erkennen [15] Im Gegensatz zu den von HIRSCHHORN *et al* [8, 11] erhobenen Befunden sahen diese Autoren [15, 16] in PHA-behandelten Kulturen eine der sauren Phosphatase analoge Aktivitätsänderung der β Glucuronidase und der α Glucosidase, eines weiteren lysosomalen Enzyms Nach 3tägiger PHA Stimulierung hatte nur die spezifische β-Glucuronidase-Aktivität das Niveau der Kontrollkulturen annähernd wieder erreicht, während die pro Zellzahl berechnete Aktivität noch deutlich über dem Ausgangswert lag Der wesentliche Unterschied zwischen den von HIRSCHHORN *et al* [8, 11] und von NADLER *et al* [15, 16] mitgeteilten Ergebnissen besteht somit darin, dass nur die zuerst genannten Autoren nach PHA Stimulierung ein unterschiedliches Verhalten der lysosomalen Enzyme saure Phosphatase und β Glucuronidase feststellen konnten Die von uns in PHA Kulturen am 3 Tag nachgewiesenen Aktivitäten der sauren Phosphatase und β-Glucuronidase sprechen wie die Befunde von HIRSCHHORN *et al* [8, 11] ebenfalls für ein unterschiedliches Verhalten beider Enzyme NADLER *et al* [15, 16] konnten auch Aktivitätssteigerungen der nicht lysosomalen Enzyme Laktat Dehydrogenase und Glukose 6-Phosphat Dehydrogenase verifizieren, denen die von uns beobachtete Steigerung der MDH Aktivität zugeordnet werden kann Die Ergebnisse unserer biochemischen Untersuchungen, die eine Neusynthese und/oder Aktivierung von saurer Phosphatase in stimulierten normalen Lymphozyten wahrscheinlich machen, werden durch die zytochemischen Befunde gestützt Die morphologisch, zytochemisch und biochemisch fassbaren Zellveränderungen liefen parallel ab und erreichten ihr Maximum am 3 Stimulierungstag

Bei CLL-Lymphozyten entsprachen die im Vergleich zu normalen Zellen verminderte Transformationsrate und DNS Synthese sowie der

verzögerte Eintritt der Reaktion auf PHA den Angaben der Literatur. Durch die zyto- und biochemischen Untersuchungen konnte wahrscheinlich gemacht werden, dass *in stimulierten CLL-Lymphozyten* eine Neusynthese und/oder Aktivierung von saurer Phosphatase stattfand. Der Aktivitätsanstieg dieses Enzyms trat dem Stimulierungsverlauf entsprechend verzögert ein. Der höchste Enzymgehalt wurde wie in stimulierten normalen Lymphozyten zum Zeitpunkt der maximalen Blastentransformation gemessen. Sowohl die zytochemischen als auch die biochemischen Ergebnisse sprechen dafür, dass das Ausmass dieser Enzym-Neubildung und/oder -Aktivierung in CLL-Blasten geringer war als in *entsprechenden Zellen von Normalpersonen*.

Die Aktivitätssteigerung der sauren Phosphatase in CLL-Blasten zeigt somit, dass *diesen Zellen die Fähigkeit eigen ist, die Stimulierung* mit PHA wie normale Lymphozyten mit Lysosomenveränderungen zu beantworten. *Da bei* unseren Versuchen normale und CLL-Lymphozyten lediglich Hinweise auf eine Neusynthese und/oder Aktivierung von saurer Phosphatase, nicht jedoch von β-Glucuronidase boten, darf angenommen werden, dass diese lysosomale Reaktion in Lymphozyten von Gesunden und Patienten mit CLL qualitativ nicht unterschiedlich ist. Ein paralleles Verhalten in stimulierten normalen und CLL-Zellen liess auch die Aktivität des nicht-lysosomalen *Enzyms MDH erkennen.*

Diese Ergebnisse lassen vermuten, dass CLL-Blasten und transformierte normale *Lymphozyten nicht nur*, wie von CLAUSEN und BOURONCLE [5] festgestellt wurde, ultrastrukturell, sondern auch funktionell weitgehend übereinstimmen. Diese Annahme wird gestützt durch Untersuchungen von RUBIN *et al.* [21–23], die in PHA-stimulierten CLL-Lymphozyten wie in normalen PHA-Blasten eine gesteigerte Neusynthese von ribosomaler RNS und eine vermehrte Ribosomenbildung nachweisen konnten. Das Maximum dieser Veränderungen wurde in CLL-Zellen jedoch wesentlich später erreicht als in [illegible]

[illegible] ge, warum der zur Reaktion auf PHA [illegible] mphozyten verzögert auf PHA anspricht. Dieses abnorme Verhalten *konnte seine Ursache in einer Störung des* durch PHA induzierten initialen «Trigger»-Mechanismus haben. Ob dabei die erwähnte Alteration des *RNS-Stoffwechsels [21–23]* oder fehlende Lysosomenveränderungen [3] eine Rolle spielen, muss offen bleiben.

Frl. R. Hinsch danken wir für wertvolle technische Assistenz. Herrn Prof. Dr. R. Gross, Direktor der Medizinischen Universitätsklinik Köln-Lindenthal, sind wir für die Überlassung von Blutproben eines Patienten zu Dank verpflichtet.

Ein Teil der hier mitgeteilten Untersuchungen wurde im Rahmen einer Inaugural-Dissertation (H. G. Aberle) durchgeführt.

Zusammenfassung

Im Gegensatz zu normalen Lymphozyten, in denen 3 Tage nach PHA-Zusatz eine maximale Blastentransformation und DNS-Synthese beobachtet wurden, ließen Lymphozyten von Patienten mit chronischer lymphatischer Leukämie (CLL) eine abgeschwächte und erst nach 5 bis 7 Tagen eintretende Reaktion erkennen. In stimulierten CLL-Lymphozyten konnte wie in normalen Lymphozyten eine Neusynthese und/oder Aktivierung von saurer Phosphatase, nicht jedoch von β-Glucuronidase wahrscheinlich gemacht werden. Die Ergebnisse sprechen dafür, dass in PHA-stimulierten normalen und CLL-Lymphozyten zum Zeitpunkt des Stimulierungsmaximums qualitativ gleichartige Lysosomenveränderungen auftreten, die jedoch quantitativ unterschiedlich sind. CLL-Blasten und transformierte normale Lymphozyten scheinen somit nicht nur morphologisch, sondern auch funktionell übereinzustimmen.

Summary

In contrast to normal lymphocytes which showed maximal blast transformation and DNA synthesis 3 days after addition of PHA, lymphocytes from patients with chronic lymphocytic leukemia (CLL) revealed an impaired and delayed reaction with a maximum after 5 to 7 days of PHA stimulation. Results of cytochemical and biochemical studies suggest that new synthesis and/or activation of acid phosphatase but not of β-glucuronidase is taking place not only in normal lymphocytes but also in CLL lymphocytes stimulated by PHA. However, in CLL lymphocytes the quantity of newly synthesized enzyme seems to be lower than in normal cells. Thus, as also shown by morphologic studies, CLL lymphocytes are able to transform into blast cells which are qualitatively similar to blast cells derived from normal lymphocytes.

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Red Cell Metabolism in Anaemia

The Oxygen Uptake in the Presence of Methylene Blue

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In red blood cells under normal conditions only a minor part of the glucose is metabolized through the pentose phosphate pathway and consequently the oxygen (O_2) uptake of the cell is low This however is known to increase in the presence of various oxidant substances and particularly of the oxidation reduction catalyst methylene blue (MB) [4] The mechanism by which this compound exerts its effect seems to depend entirely on its reduction by reduced triphosphopyridine nucleotide (TPNH) therefore the O_2 uptake of the cell in the presence of MB is likely to reflect chiefly the efficiency of the oxidative sequence of the pentose phosphate pathway, i e its ability to generate TPNH

So far O_2 uptake in the presence of MB has not yet been fully investigated in anaemic conditions OKA and PURANEN [13] in 30 unselected cases of anaemia have found that this red cell metabolic activity was higher than normal and was correlated with the haemoglobin level but not with the number of reticulocytes, that are known to display a higher O_2 uptake in the presence of MB than old erythrocytes [6] BRABEC *et al* [2, 3] reported a significantly higher than normal mean value in a group of 22 patients suffering from autoimmune haemolytic anaemias and normal or slightly increased values in 6 cases of paroxysmal nocturnal haemoglobinuria (PNH) Similar results were obtained in this laboratory in 51 cases of autoimmune haemolytic anaemias [20] and in 3 cases of PNH [18]

We have determined the red cell O_2 uptake in the presence of MB in 248 patients suffering from anaemia of different cause and have correlated the metabolic activity with some haematological parameters The aim of the present paper is to report the results obtained

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We have determined the red cell O_2 uptake in the presence of MB in 248 patients suffering from anaemia of different cause and have correlated the metabolic activity with some haematological parameters The aim of the present paper is to report the results obtained

Materials and Methods

Two hundred and forty-eight patients – 117 male and 131 female – were included in the study the range varied from 7 to 75 years The distribution of the cases according to the cause of anaemia is reported in table I diagnosis was based on the clinical picture and laboratory investigations The control group consisted of 62 healthy blood donors and members of the hospital staff

Blood was drawn with heparin and used within 6 hours of collection Routine haematologic investigations were performed using standard techniques. The red cell O_2 uptake in the presence of MB was performed by the manometric Warburg technique according to OKA and PURANEN [13] and expressed as µl O_2/mg dry weight/hour Statistical analysis of the data was performed using either the one-way model of the analysis of variance, or SCHEFFÉ's method [17] or the correlation coefficient r according to the information required

Results

Results are reported in table I and figure 1 Statistical analysis indicates that when the patients are considered all together the red cell O_2 uptake in the presence of MB is significantly higher than normal ($P<0.01$) Comparing 11 of the 13 considered groups of patients with the control group (groups No 4 and 8 are excluded because numerically small), it appears that a significant increase ($P<0.05$) of this metabolic activity can be found only in autoimmune haemolytic anaemias, thalassemic syndromes, iron deficiency anaemia and chronic renal insufficiency Since these latter comparisons were made using a cautious statistical approach (SCHEFFÉ's method at the 5% level of significance), the possibility remained that the other conditions, although different from normal, could not be shown as such with the test used Therefore employing the same statistical method an additional comparison was made between the control group and a pool of all the anaemic conditions which had previously appeared not significantly different from normal, and a significant difference was indeed found ($P<0.05$) The subsequent step was then to test, within the pool, each of the anaemic conditions against each of the others This time no significant difference was observed suggesting that in fact the conditions considered had a higher than normal O_2 uptake in the presence of MB although not sufficiently so to be significantly different from normal when considered separately yet sufficient to make apparent such a difference when considered together

Insofar as the results of the correlations investigated are concerned, the individual correlation coefficients are reported in table II Taking

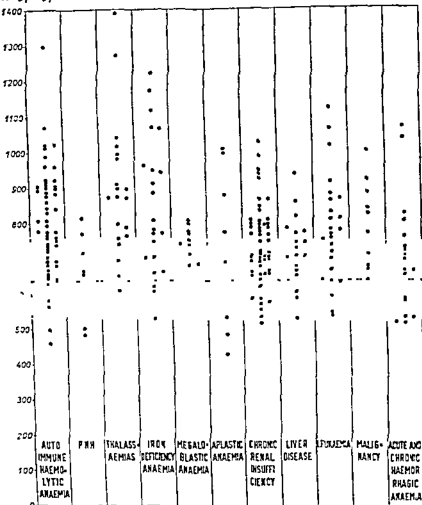


Fig 1 Red cell O_2 uptake in the presence of methylene blue in the anaemic patients investigated (2 cases of sideroblastic anaemias and 1 of hereditary spherocytosis are not reported) Each dot represents a single case The lines indicate mean value ± one standard deviation obtained *in normal subjects*

[illegible] standard deviation of red cell O_2 uptake in the presence of methylene blue and of some haematological parameters in the anaemic conditions investigated

Anaemic conditions	Number of subjects	O_2 uptake in the presence of MB QO_2[1]	Haemoglobin g/100 ml	Reticulocytes %	MCV μm^3	MCH pg	MCHC %
Autoimmune haemolytic anaemia	54	0.806±0.148	8.80±2.49	8.21±2.75	99.22±12.24	30.72±1.99	31.57±3.74
Paroxysmal nocturnal haemoglobinuria	7	0.656±0.122	6.18±3.07	4.13±4.18	111.60±19.82	32.10±5.31	28.80±3.70
Thalassaemia	20	0.888±0.189	9.05±1.99	1.76±1.99	74.65±10.67	20.80±3.12	27.90±2.75
Hereditary spherocytosis[2]	1	0.835	9.10	1.87	99.63	33.57	33.70
Iron-deficiency anaemia	22	0.818±0.192	7.85±1.16	1.60±1.18	80.95±12.31	20.81±3.96	25.72±2.51
Megaloblastic anaemia	10	0.750±0.031	8.01±2.51	1.91±2.10	102.70±20.72	32.60±5.67	31.90±2.21
Aplastic anaemia	8	0.725±0.225	8.25±2.09	0.64±0.73	108.00±21.81	33.50±7.38	31.29±2.12
Sideroblastic anaemia[2]	2	0.781–0.973	8.30–7.40	0.60–2.40	101.50–101.51	30.74–32.17	31.20–33.63
Anaemia associated with chronic renal insufficiency	53	0.739±0.109	8.61±2.11	0.99±0.77	93.88±9.52	29.20±3.40	30.83±2.66
Anaemia associated with liver disease	18	0.723±0.091	9.66±1.71	1.63±1.60	100.11±12.11	32.66±4.12	30.00±1.80
Anaemia associated with leukaemia	25	0.778±0.144	8.51±2.21	0.85±0.52	90.08±14.18	28.48±1.24	31.60±4.24
Anaemia associated with malignancy	10	0.793±0.144	8.64±2.02	1.51±1.36	94.70±9.59	28.60±4.15	29.90±11.43
Acute and chronic haemorrhagic anaemia	18	0.699±0.158	7.96±2.43	3.42±2.36	89.50±13.19	26.11±5.13	28.83±2.62
Total	248	0.777±0.151	8.56±2.21	3.39±5.62	93.97±15.51	28.45±5.80	30.15±3.52

[1] Normal values = 0.637 ± 0.105
[2] Individual values given for this group

Table II Correlation coefficients (r) between red cell O_2 uptake in the presence of methylene blue and some haematological parameters obtained in the anaemic conditions indicated

Anaemic conditions	Haemoglobin level	Reticulocyte percentage	MCV	MCH	MCHC
Autoimmune haemolytic anaemia	0.03	−0.09	−0.15	−0.15	0.14
Paroxysmal nocturnal haemoglobinuria	0.16	0.29	−0.41	−0.07	0.47
Thalassaemia	−0.45[2]	0.33	−0.39	−0.60[3]	−0.35
Iron-deficiency anaemia	−0.41[1]	−0.07	−0.24	−0.43[1]	−0.42[1]
Megaloblastic anaemia	−0.37	0.18	0.24	0.11	−0.30
Aplastic anaemia	−0.22	0.36	0.45	0.21	−0.65[1]
Anaemia associated with chronic renal insufficiency	−0.33[1]	0.09	0.26	0.03	0.09
Anaemia associated with liver disease	−0.37	0.38	−0.24	−0.07	−0.39
Anaemia associated with leukaemia	−0.28	0.43	−0.39[1]	−0.46[1]	0.16
Anaemia associated with malignancy	−0.40	−0.03	−0.13	−0.09	−0.09
Acute and chronic haemorrhagic anaemia	−0.40	−0.33	−0.42	−0.35	−0.27
Total	−0.85[3]	0.02	−0.24[3]	−0.30[3]	−0.11

[1] $r \neq 0$ at a significance level at 0.05.
[2] $r \neq 0$ at a significance level at 0.01.
[3] $r \neq 0$ at a significance level at 0.001.

into account only the correlations with a coefficient higher than 0.70 it was found that: (1) Considering the patients all together, the red cell O_2 uptake in the presence of MB appeared to be negatively correlated with the haemoglobin concentration ($r = -0.85$; $P < 0.001$). (2) Considering each anaemic condition by itself, no correlation resulted between the metabolic activity and the corpuscular indices determined.

It is noteworthy that in the above two cases no correlation was found between the red cell metabolic activity and the percentage of reticulocytes.

Discussion

This investigation confirms and extends previously reported results [2, 3, 13, 18, 20] that red cell O_2 uptake in the presence of MB is increased in anaemia. The difference from normal was significant for the groups of thalassaemias, iron-deficiency anaemia, autoimmune haemolytic anaemias and chronic renal insufficiency.

In thalassaemias and in iron-deficiency anaemia, the red cell metabolic activity was markedly increased in spite of the presence in the peripheral blood of only a slightly higher mean percentage of reticulocytes. In thalassaemias the survival of red cells is decreased and this results in a lower mean age of circulating erythrocytes, and may explain the increase in red cell O_2 uptake in the presence of MB. In iron-deficiency anaemia, on the contrary, studies of erythrocyte life-span have given conflicting results, the survival of red cells having been reported as normal [5, 10, 14, 21] or decreased [8, 11, 15, 22]; moreover other clinical and laboratory features characteristic of anaemias with shortened red cell survival are often lacking in this condition [11]. Therefore it seems difficult to relate the high erythrocyte O_2 uptake in the presence of MB found in this disorder to an increase in the number of circulating young cells. The abnormality may rather be explained as a compensatory mechanism on the part of malformed red cells, as suggested by MACDOUGALL [12] for some enzyme abnormalities found in this condition. The above explanation is also supported by the results of serial studies carried out in iron-deficient patients during iron therapy: in 2 patients it was observed that an increase in haemoglobin and serum iron values during therapy was accompanied by a decrease in red cell O_2 uptake in the presence of MB in spite of the increase in reticulocyte percentage. A compensatory mechanism could similarly act in determining the increased red cell metabolic activity observed in patients with chronic renal insufficiency. In fact on the one hand in these patients the higher the red cell metabolic activity was found to be, the worse the kidney function; on the other hand in these patients there was no evidence of excessive red cell destruction and the red cell glucose-6-phosphate dehydrogenase activity, a good expression of cell age [16], was within normal limits (unpublished results).

In autoimmune haemolytic anaemias the increase in red cell O_2 uptake in the presence of MB (like that of other enzymatic and

metabolic activities [see 20]) can be an expression of the high percentage of reticulocytes in peripheral blood On the contrary in the patients with PNH, another haemolytic condition where the mean reticulocyte count was approximately twice normal, the red cell O_2 uptake in the presence of MB was within normal range This finding suggests a relative impairment of the red cell metabolic activity in PNH In this disorder there is an erythrocyte membrane defect and acetylcholinesterase, an enzyme located in the red cell stroma, is typically diminished [1, 7, 18] Since red cell O_2 uptake in the presence of MB reflects the metabolism of the cell membrane [13], its relative reduction might be a further biochemical manifestation of membrane abnormality This possibility is supported by the finding that normal human red cells with membrane alteration caused by *in vitro* treatment with chemically unrelated substances show a significant decrease of O_2 uptake in the presence of MB [9, 19]

In conclusion, the results reported here indicate that an increase in red cell O_2 uptake in the presence of MB is an accompanying feature of anaemia and it occurs with particular intensity in some anaemic conditions probably as a consequence of a decrease in the mean age of circulating erythrocytes and/or an unfavourable environment for red cell formation or survival This latter causal factor is still ill defined and in the demain of speculation, and further studies are warranted to define in biochemical terms the compensatory mechanisms that red cells develop in particular conditions

Summary

sufficiency The pathogenesis of this finding is discussed and t is suggested that the metabolic abnormality occurs as a consequence of a decrease in the mean age of circulating erythrocytes and/or an unfavourable environment for red cell formation or survival.

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Thalassaemia and High F-Gene in Aleppo

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Studies of the incidence of abnormal haemoglobins in Syrians have been few and so far only three reports have been published [6, 12, 14]. However, these reports have centred mainly on the investigation of immigrant Syrians.

The presence of sickle-cell haemoglobin in the inhabitants of a village near Damascus had been reported by Shahid and Haydar [12]. Aksoy [1, 2] had previously demonstrated the presence of the sickle-cell gene in the Southern part of Turkey. Furthermore, thalassaemia and haemoglobins S and E were detected in Southern Turkey amongst the Eti-Turks [3]. The Eti-Turks, an Arabic speaking community of obscure origin, are believed to have emigrated to Southern Turkey from Egypt and Syria.

During June 1969 we examined 25 blood specimens from children admitted to the National Hospital for Children, Aleppo, Syrian Arab Republic. The results are incorporated in this paper.

Material and Methods

The material consisted of 25 heparinized blood samples obtained from children admitted

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Results

In all 35 individuals examined, no abnormal haemoglobin was detected. However, table I shows the haematological findings in 15 of the children investigated. As is seen, the majority of the blood samples showed elevations in the percentage of haemoglobin A_2 which was associated with a varying degree of raised foetal haemoglobin. Peripheral blood smears showed anisopoikilocytosis together with microcytosis and occasional target cells. The diagnosis of β-thalassaemia minor was made in 12 cases and indeed family studies showed that one or the other of the parents had a rise in the percentage of haemoglobin A_2.

Three cases (No. 2, 3 and 30) showed elevations in foetal haemoglobin levels which were in excess of those observed with thalassaemia minor. Family studies in these cases showed that in case No. 30 both parents had raised haemoglobin A_2 levels and the morphology of the erythrocytes was in conformity with that of thalassaemia minor. The patient had been admitted to hospital several times during the past year and received 3 blood transfusions. A tentative diagnosis of thalassaemia major was made in this case.

Table I. Haematological findings in 15 children at the national hospital Aleppo

Case No.	Sex	Age years	Hb g%	Adult haemoglobins	% Hb A_2	% Hb F
2	F	3	8.2	$A+A_2$	1.5	21.0
3	M	3	7.8	$A+A_2$	2.0	80.0
5	M	7	10.5	$A+A_2$	3.5	3.0
7	F	10	11.6	$A+A_2$	3.2	3.0
8	M	10	13.5	$A+A_2$	4.0	4.6
9	F	13	9.7	$A+A_2$	5.6	6.6
10	F	11	9.8	$A+A_2$	5.8	6.3
11	F	2	11.5	$A+A_2$	3.6	2.2
12	M	3	9.7	$A+A_2$	3.0	3.0
15	M	9	7.6	$A+A_2$	5.0	2.1
17	F	10	11.6	$A+A_2$	4.1	3.0
18	F	3½	10.5	$A+A_2$	3.2	4.0
19	M	1	9.7	$A+A_2$	4.0	6.5
27	M	1½	14.0	$A+A_2$	3.6	5.4
30	M	2½	6.0	$A+A_2$	4.5	9.0

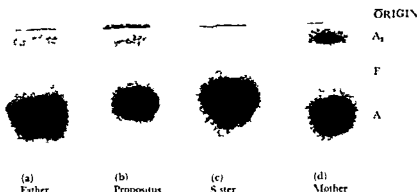


Fig 1 Paper electrophoresis of haemoglobins in the family of case No 2

Table II Haematological findings in the family study of case No 2

Subject	Age years	Hb g%	Adult haemoglobins	% Hb A_2	% Hb F
Propositus	3	8 2	A + A_2	1 5	21
Father	30	15 0	A + A_2	1 8	10 3
Mother	28	11 6	A + A_2	3 5	1 5
Sister	4½	9 5	A	0	85

The results obtained by examining the family of case No 2 are shown in table II and figure 1 The father had no evidence of thalassaemia minor but instead he showed persistence of foetal haemoglobin The mother on the other hand showed both the biochemical and haematological criteria of thalassaemia minor An elder sister when examined, revealed that 85% of her haemoglobin was of the foetal type Furthermore, no haemoglobin A_2 could be detected electrophoretically (fig 1) Similar results were obtained in the family of case No 3 The father had persistence of foetal haemoglobin, the mother had thalassaemia minor and a younger sister, aged 8 months, had a foetal haemoglobin level of 90% Staining for foetal haemoglobin after acid elution [9], confirmed the diagnosis

Discussion

The results obtained in this pilot survey indicate the presence of β thalassaemia in Northern Syria. This would be expected since this abnormality is common in the Mediterranean Countries.

The detection of persistent high F gene of the type described in West and Central Africa [5, 8, 11] is interesting. The high F gene occurs both in Africa and around the Mediterranean [10]. It is probable that this gene might play a role in the protection of affected individuals particularly infants against infection with malaria. Out of 3.6 millions living in Syria, 1.15 millions live in malarious areas [15]. This might explain a possible advantage for the persistent high F gene in Syria.

Persistence of foetal haemoglobin into adult life is a genetic entity unassociated with any other haematological abnormality [8]. However, the cases detected in the present investigation show a mild to severe anaemia (tables I and II). Indeed cases No. 2 and 3 were admitted to hospital for this same cause and were apparently diagnosed as thalassaemia major on the grounds of finding raised foetal haemoglobin levels. However, once the diagnosis of persistent high F gene was made, the patients made a reasonable recovery with iron therapy. It is suggested that the high F gene might complicate a co-existing iron deficiency anaemia in childhood.

It is of interest to note that the sister of case No. 2 had no detectable haemoglobin A_2 on electrophoresis (fig. 1). This is a finding usually elicited with homozygotes of the high F gene. However, the family study excluded the occurrence of homozygosity (table II). It is therefore tempting to postulate that the high F gene may depress the synthesis of the δ-chains more than the β chains. If this is so, then the anaemia observed in the cases reported here might have been the result of a co-existing β thalassaemia minor the biochemical detection of which has been missed as a result of depressed synthesis of haemoglobin A_2.

Acknowledgements. We are grateful to Dr. A. R. AKTA, Dean, Faculty of Medicine, Aleppo for the help and enthusiasm he showed; to Drs. W. SAKAI and B. BAZIRBASHI for assistance; to Mr. T. R. DAVIS, Supervisor, Trainee Technician Unit, University of Khartoum, for photographing the electrophoretograms and to MOHAMED CHATI for technical assistance.

Summary

Central Africa was detected The significance of these findings is discussed

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A Particularly Sensitive Modification of the Peroxidase Reaction

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One of the most usual methods for the demonstration of peroxidase in blood and bone marrow smears is that of GRAHAM and KNOLL, several modifications of which were developed by UNDRITZ [5, 6]. However, very weak activities are not readily demonstrable by these methods. Nevertheless, such faint activities at times can be of definite diagnostic importance. Therefore, a further modification of a procedure already reported [2] will be described which allows to detect even minimal peroxidase activities. Accordingly, the method is particularly suitable for the identification of weakly peroxidase-positive atypical myeloid cells, which may react negatively with less sensitive methods.

Method

Thin, thoroughly prepared air dried blood or bone marrow smears not older than 1 or 2 days are fixed in methanol-formalin (1: 9) [1] for 2–3 sec. Immediately after fixation they are rinsed in tap water and allowed to dry. *Reaction medium:* 200 mg of benzidine are dissolved in 4 ml acetone and 4 ml dimethylsulfoxide. 32 ml of destilled water and 0.08 ml of 3% H_2O_2 are added. The preparations then are exposed to the filtrated medium for 10 min at room temperature. During the incubation period the medium should be kept in permanent movement, e.g. by means of a mechanical shaker. After incubation the smears are rinsed in tap water and counterstained with hemalum. Finally, the air-dried smears are mounted in eukitt or any other suitable medium.

Result: Nuclei blue, positive structures brownish to blackish.

Discussion

Recently, SCHAFFER and FISCHER [4] reported on a peroxidase reaction which can be applied both on smears and paraffin sections

Our intention was to elaborate a procedure by which particularly weak peroxidase activities can be demonstrated. By this means informations about the character of certain cells, e.g. of unclassifiable immature leukemias, may be achieved, thus allowing to recognize myeloid leukemias easier than would be possible by less sensitive peroxidase methods.

Two facts have been taken into account with the described method. These are the very marked inhibitory effect of alcohol on the peroxidase activity and the omission of a cytoplasmatic counterstain in order to facilitate the recognition of weakly positive reactions.

Considering the first fact, instead of the fixative used with the GRAHAM-KNOLL method which contains almost 90% alcohol, the medium of KAPLOW [1] with only 10% alcohol was applied. In addition, the fixation time was limited to 3 sec, which is completely sufficient to render a satisfactory structural preservation, provided that thin smears are used. Finally in contrast to the GRAHAM-KNOLL method our incubating medium is free from alcohol. So the almost complete avoidance of alcohol resulted in a good preservation of the enzyme activity.

As was to be expected from these technical precautions the modification revealed the following results (fig. 1). The eosinophilic and neutrophilic cell series were very strongly positive, the reaction product



Fig. 1. One neutrophilic granulocyte and 5 monocytes. Peripheral blood smear. Peroxidase reaction. Extremely strong reaction of the neutrophil, the nucleus of which is completely covered with the reaction product. Note the very strong activities of some of the monocytes and the distinct granular deposition of the reaction product. × 1400.

often completely covering the nuclei. Most of the monocytes showed a moderate or even a rather strong activity, the reaction product being visible as fine or coarse brownish granules, whereas with the GRAHAM-KNOLL method many monocytes are negative and relatively few weakly positive. As there is no cytoplasmatic counterstain, low grade activities can easily be detected both in the original preparations and in microphotographs.

We observed leukemic immature cells, which were negative with the naphthol AS-D chloroacetate esterase reaction and therefore could not be identified as myeloid cells. However, applying our modification of the peroxidase reaction these cells could be easily recognized as myeloid ones by their distinct activity [3]. This clearly shows that both the peroxidase reaction and the naphthol AS-D chloroacetate esterase reaction are apt to the demonstration of myeloid cells. However, they should not be understood as alternatives revealing nearly identical results as some believe. Therefore, if a careful cytochemical analysis of blood and bone marrow cells, especially in leukemias, is intended, in addition to other methods both reactions must be applied.

Summary

A modification of the peroxidase reaction is described, in which the inhibitory effect of alcohol is restricted to a minimum. This and the omission of a cytoplasmatic counterstain allow to clearly demonstrate even very scanty activities which can be of important value with respect to cytological and clinical diagnostic problems.

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Multiple Myeloma in One of a Pair of Monozygotic Twins

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Multiple myeloma is generally regarded as a malignant process in which a clone of plasma cells autonomously proliferates and generates a large quantity of an antigenically homogeneous globulin, usually referred to as an M-protein or a monoclonal protein [24] It has been further suggested that the abnormal monoclone develops through random mutation from the normal polyclonal population [8, 10]

Thus far, to our knowledge, 12 familial occurrences of this disease have been reported leading to speculation that hereditary factors are important in the development of this disease [2, 7, 15] On the other hand, a cluster of myeloma recently reported suggests an environmental cause [27] It appears that the observation of a case of multiple myeloma in one of a pair of monozygotic twins may be of considerable value in assessing the relative importance of genetic or environmental factors in the causation of the disease This report is concerned with such a case, the first reported, to our knowledge Our patient and his twin brother who does not have myeloma both display cytogenetic abnormalities

Case Report

[illegible]

[illegible] age [illegible] with minimal residual weakness of the left foot Physical examination revealed an obese pale-appearing male complaining of back pain The mucosae were pale and the thoracic spine revealed marked kyphosis with moderate percussion tenderness The skin was dry hair coarse, and the deep tendon reflexes had a slow return The thyroid was not palpable Laboratory investigation revealed a hemoglobin

of 8.7 g%, hematocrit 26%, WBC 5,800/mm³ and platelet count 405,000/mm³. Erythrocyte sedimentation rate 131 mm/h, BUN 28 mg%, the serum uric acid 5.9 mg%, calcium 9.8 mg%, phosphorus 3.9 mg%, respectively. The total protein was 8.9 g% and serum electrophoresis showed a prominent M spike in the γ-region representing 1.2 g% of protein. (Unfortunately the immunoglobulin class of the M spike was not determined this time. A quantitative immunodiffusion of the serum in March, 1967, when the M protein had disappeared showed IgG 900 mg%, IgA 260 mg% and IgM 35 mg%.) The urine was negative for Bence-Jones protein by the method of SNAPPER and ORES [21] but urine electrophoresis showed a small monoclonal peak in the γ-area. A bone marrow examination revealed 24% plasma cells, and a skeletal survey showed generalized osteoporosis with compression fractures of 2 thoracic vertebrae. The PBI was 1.4 μg% and a 24 hour radio- [illegible]

the dosage of cyclophosphamide was reduced to 50 mg daily because of bone marrow suppression and he has since been maintained on this drug. The patient has been followed regularly and continuous improvement was noted (fig. 2). Although the patient is still forced to wear a back brace for support, the M protein and marrow plasma cells have disappeared and hemoglobin has improved. Serial bone X rays have remained unchanged. In June 1970, almost 7 years after the initial diagnosis of myeloma, the bone marrow plasma cell was 6% and there was no M protein on serum electrophoresis.

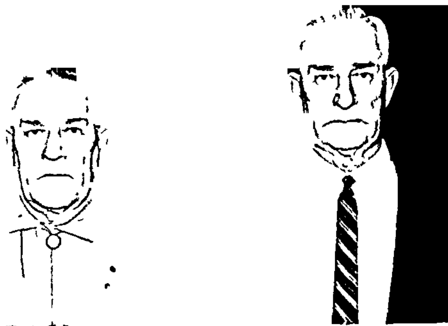


Fig. 1. Left: patient (Clair). Right: patient's healthy twin (Clifford). This picture was taken in December 1965.

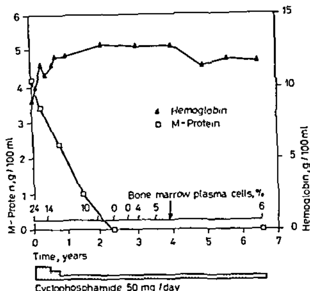


Fig 2 Good response to chemotherapy as indicated by laboratory tests

The patient's twin (Clifford H, fig 1) was first seen by us on December 14, 1965 His history, physical examination, hemogram, and serum electrophoresis were normal On following visits he had remained healthy with no signs or laboratory evidence of multiple myeloma or myxedema A bone marrow examination and serum electrophoresis were again normal in June 1970

allowed to sediment at room temperature until the cell rich plasma could be separated.

used in control cultures had been prepared in our laboratory by the method of B[illegible] *et al* [4] and contained 2 8 mg of protein/ml The results are described in table I. It will be noted that no augmentation of thymidine uptake occurred when the cells of the two brothers were cultured together The cells were capable of stimulation with PHA or when cultured in the presence of cells from unrelated individuals.

Table I Mixed leukocyte reaction and PHA stimulation of the leukocytes from the patient, his healthy twin and 2 unrelated control subjects. Results are expressed by the tritiated thymidine uptake in cpm by cultured leukocytes The mixed culture of patient's and his twin's leukocytes reveals no increase in tritiated thymidine uptake over those obtained by culturing the cells alone

Subject	Mixed with cells from subject			Control cultures	
	Twin	Control 1	Control 2	No PHA	PHA
Patient (Clair)	64	724	627	33	17,606
Twin (Clifford)	–	757	736	70	12,771
Control 1	–	–	1,205	280	18,551
Control 2	–	–	–	130	29,579

Table II The distribution of the chromosomal counts of bone marrow cells and leukocytes from patient (Clair) and his healthy twin brother (Clifford)

No of chromosomes	Unclear	44	45	46	47	>47
Patient (Clair)						
Bone marrow	10	1	0	3	2	0
Blood	14	4	20	89	9	2
Twin (Clifford)						
Bone marrow	24	0	7	5	0	5
Blood	6	2	7	105	5	0

Many of the cells from both of the twins that were not clear enough to be accurately counted ('unclear') fell distinctly into a hypodiploid range of below 43 elements In Clair, only 2 bone marrow cells could be karyotyped, one of which was normal and the other showed 47 elements with an extra element of probable G group In his blood leukocytes, 4 cells with 47 chromosomes karyotyped had an extra element about the size of No 19 22 elements (fig 3) In addition, many cells with random changes were present In Clifford, 4 of the 16 cells karyotyped had 47 chromosomes, each with an extra element similar to a large G-chromosome in size and shape (fig 4)

Cytogenetic Studies Chromosome studies were done using slight modifications of the method of MOORHEAD *et al* for short term peripheral blood cultures [12] and of the method of TJIO and WHANG for direct, air-dried bone marrow preparations [23]

Both the patient (Clair) and his healthy twin (Clifford) had aneuploidy in the blood and bone marrow cells, although it was more pronounced in the former (table II) The hypodiploid cells with 45 elements showed no consistent loss in either Clair or Clifford, but the hyperdiploid cells having 47 elements in both partners consistently possessed an extra element of the size corresponding to a large G chromosome The representative karyotypes from hyperdiploid (47) cells are illustrated in figure 3 (Clair) and figure 4 (Clifford) There were several hyperdiploid cells with chromosomes missing and extra elements present at

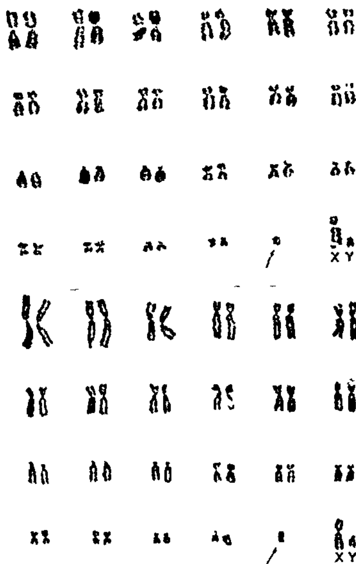


Fig 3 Karyotype of a hyperdiploid (47) cell from the patient's (Clair) blood leukocytes The arrow indicates an extra chromosome of the size corresponding to a G-chromosome.

Fig 4 Karyotype of a hyperdiploid (47) cell from the patient's healthy twin (Clifford). The arrow indicates a small extranumerary autosome similar to the one noted in the patient (fig 3)

random. More cells of this type were evident in [illegible] than in [illegible]. The chromosomes of both men displayed an unusual degree of telomere association and general stickiness. Only in [illegible] were chromatid breaks observed.

Discussion

Although the two brothers were often confused by their parents and close friends when they were young, have identical blood groups and do not stimulate in the mixed leukocyte reaction, we have not unequivocally established that they are identical. We do not think it advisable to place a skin graft from the patient on his healthy brother. Nor do we wish to discontinue the successful cyclophosphamide treatment in the patient prior to grafting him with skin from his healthy twin. It can be calculated that the chances of two siblings with unrelated parents having the identical blood types found in these two brothers is about 3.5% [19]. The chance of two siblings with unrelated parents having an identical HL-A locus as revealed by the *mixed leukocyte reaction is 1 in 4. Thus the probability that these* twins are identical is approximately 99%.

It could be argued that the initial marrow plasmacytosis and monoclonal protein were the result of a benign monoclonal gammopathy associated with myxedema, and that the thoracic spine changes were secondary to poliomyelitis at age 20. The large quantity of the monoclonal protein, the progressive nature of bone disease leading to osteoporosis, compression fractures, and a severe kyphosis coupled with the remarkable response to an alkylating agent, are inconsistent with this hypothesis and strongly suggest that the underlying process is multiple myeloma. To our knowledge, only one other case of myeloma associated with myxedema has been described in the literature [3]. A case of primary amyloidosis with mild bone marrow plasmacytosis, myxedema and carpal tunnel syndrome has been described [26]. WALDENSTRÖM found a serum with weakly positive anti-thyroglobulin antibody activity among 11 myeloma sera [25]. In our series of 87 cases of multiple myeloma about 10% of those afflicted with the disease had a previous history of thyroid disorder or were found to have thyroid dysfunction at or after the diagnosis of myeloma[1].

SELIGMANN is of the opinion that the incidence of paraproteinemia among blood relatives of patients with myeloma is higher than is

[1] Unpublished data.

statistically expected [17], and thus far, to our knowledge, 12 familial instances of multiple myeloma have been reported McKUSICK judges the evidence to be sufficiently strong to warrant the inclusion of myeloma in his list of autosomal recessive disorders [11] This report is the first to describe a case of multiple myeloma noted in one of a pair of identical twins Although SNAPPER and KAHN in their review of myeloma stated that they had seen a patient with myeloma whose identical twin was healthy [20], there has been no detailed description of the case A case similar to ours has been reported by SPENGLER *et al* with respect to WALDENSTROM's macroglobulinemia [22] Cultures of their patient s blood and bone marrow demonstrated two cell lines with 47 chromosomes, one with a characteristic extra chromosome of A group and the other with a small metacentric extra chromosome similar to the No 20 autosome The chromosomal pattern of the blood from the healthy partner was numerically normal but showed an abnormal frequency of chromatid breakage They postulated that the abnormal chromosomal patterns were acquired changes, and that the extra chromosomes of the size of the No 20 element were derived from a cell line containing extra chromosomes of the A group through the mechanism of deletion SIEBNER *et al* considered the presence of a supernumerary small autosome, especially of group F, as a sign of higher degree of malignancy in paraproteinemic neoplasia [18]

The result of the chromosome analysis in our case was most un usual Both the myeloma patient and his phenotypically normal twin had, in addition to normal cells, aneuploid cells with numerous inconsistent abnormalities, and one consistent abnormality (a small extra chromosome in size to an F or G group element) in a low percentage of cells The difference between the two was the greater percentage of abnormal cells found in the patient

Although the cyclophosphamide that the patient had taken for 7 years might have caused additional chromosomal changes, the abnormalities noted in both twins are similar enough to suggest some common factors It is possible that these changes are congenital but entirely unrelated to the patient's myeloma The presence of an extra numerary autosome may or may not be associated with congenital deformities [6, 13, 14] Another possibility is that, although these chromosomal abnormalities are congenital changes and predispose an individual to paraproteinemia, additional factors may be required for the actual neoplasia to evolve The presence of other varieties of

abnormal chromosomes, however, suggests that the entire picture is not solely of congenital origin. Thus the third possibility is that these abnormal patterns are acquired changes and are related to the occurrence of myeloma in the patient, and that the healthy twin (Clifford) is en route to developing a form of paraproteinemic malignancy.

The chromosomal changes seen in myeloma are variable, and the occurrence of an extra chromosome of group A is far less frequent than in Waldenström's macroglobulinemia [5, 9]. Most of the abnormal chromosomal patterns reported in myeloma were obtained from PHA stimulated peripheral blood cells in non leukemic myeloma patients. Neither of our twins at the time of cytogenetic study had many plasma cells in the peripheral blood.

The labeling index of plasma cells in patients with IgG myeloma unassociated with plasma cell leukemia is of the order of 3% after a 4 hour incubation with tritiated thymidine [16]. Making an assumption that the mitotic index for the plasma cells of our patient and his brother is similar to that in IgG myeloma cells, it is clear that the number of hyperdiploid cells with the chromosome abnormality exceeds the number accounted for by mitosis in the observed number of marrow plasma cells. The finding of the anomaly in the PHA stimulated peripheral blood cultures in which no cells were seen resembling plasma cells suggests that the cytogenetic defect exists in a lymphoid cell line, possibly a precursor of the malignant plasma cells.

Although we have focused on the presence of myeloma in our patient in contrast to his twin, we also recognize the myxedema was a discordant finding. Whether both disease developed as a result of the same genetic accident or an environmental factor is speculative. Further follow up of the healthy twin is, of course, planned.

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Summary

A case of multiple myeloma in one of a pair of monozygotic twins is described. Cytogenetic studies of the blood and bone marrow cells revealed that both of the twins have, in addition to normal cells, aneuploid cells with numerous inconsistent abnormalities, and one consistent abnormality (an extra chromosome of a size corresponding to No. 19 [illegible] elements) in a low percentage of cells. The significance of this case and the cytogenetic findings with respect to the role of genetic and environmental factors in the pathogenesis of myeloma is discussed.

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Thrombozytopenie beim experimentellen HIPA Plasmozytom der BALB/c Maus

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Das transplantable HIPA Plasmozytom ist eine mesenteriale Neoplasie der BALB/c Maus, die regelmassig von einem terminalen hämorrhagischen Aszites begleitet ist [22, 23, 24] In dieser Arbeit wird das quantitative Verhalten der Thrombozyten im peripheren Blut beim transplantablen HIPA Plasmozytom wahrend seiner Evolution – einerseits in Abhangigkeit von der transplantierten Tumorzellzahl, anderseits von der Menge der ubertragenen Aszitesflussigkeit – untersucht und zur Frage Stellung genommen, welche Beziehung zwischen Thrombozytopenie und hämorrhagischem Aszites besteht.

Versuchsanordnung

Kontrolltiere

Dreissig gesunde Mäuse verschiedenen Alters und Geschlechts vom Stamm BALB c aus eigener Zucht dienten zur Bestimmung der normalen Thrombozytenwerte

HIPA Tumor Spendertiere

Mause der 82 und folgenden Transplantationsgenerationen wurden verwendet (Tab. I) Die Mäuse wurden mit Äther getotet. Die Bauchhohle wurde eroffnet und die Aszitesflüssig keit mit einer sterilen Spritze entnommen

Empfängertiere

Vorversuche mit konstanter Tumorzellzahl Bei 50 BALB c Mausen verschiedenen Alters und Geschlechts wurden konstante Tumorzellzahlen transplantiert

Empfängertiere	konstante transplantierte Tumorzellzahl × 10⁶	durchschnittliche Thrombozytenzahl × 10³
10	0 5	688
10	2 0	704
10	10 0	690
10	20 0	684
10	40 0	697

Tabelle I

HIPA Tumor Spendertier	Tumorgeneration	transplantierte Zellzahl $\times 10^6$/ml	Anzahl Empfängertiere	Geschlecht	Gewicht g	Thrombozyten-Kontroll-Tag
A	83	0,21	10	m	20	1
B	83	1,31	10	w	23	1
C	82	2,25	10	m + w	35	1
D	82	43,7	10	m	18	1
E	83	6,65	10	m + w	27	1
F	84	10,7	10	m	30	1
G	84	1,45	10	w	18	2
H	84	13,5	10	m	34	2
I	85	1,9	10	m + w	23	2
J	85	3,35	10	m	19	3
K	85	0,47	10	m	35	3
L	85	1,45	10	w	34	4
M	83	7,0	4	m	38	4
N	83	9,6	10	w	24	4
O	83	0,5	10	m	34	5
P	83	0,99	10	m	20	5
Q	83	5,1	10	m	30	6
R	86	8,8	10	w	30	6
S	85	8,0	10	m	25	7
T	85	0,99	10	m	35	7

Das Ausmass der Thrombozytopenie ist unabhängig von der Zahl der transplantierten Tumorzellen wie auch vom Alter und Geschlecht der Mäuse. In der eigentlichen Versuchsserie wurde deshalb immer 1 ml Aszites unverdünnt i.p. inokuliert und die entsprechende Zellzahl nachher ermittelt.

Versuche mit variierender Tumorzellzahl (Tab. I). Für je 10 ml Aszitesflüssigkeit der Spendertiere (A, B, C ... bis T) wurde eine Gruppe von 10 gesunden BALB/c Mäusen verschiedenen Alters und Geschlechts aus dem gleichen Inzuchtstamm herangezogen. Jedem Empfängertier wurde 1 ml Aszitesflüssigkeit (variierende Tumorzellzahl s. Tab. I) unverdünnt i.p. inokuliert in Äther-Kurznarkose.

Tumorzellzählung

Die in einem ml Aszitesflüssigkeit enthaltenen Zellen wurden mit Hilfe der Türkschen Zählkammer nach der Leukozyten-Methode gezählt [16].

Thrombozytenzählung

Die Thrombozytenwerte wurden nach der modifizierten Methode von FEISSLY und LÜDIN bestimmt [16]. Blut wurde aus der Schwanzarterie der Maus in Äthernarkose mit einer Erythrozytenpipette bis zur Marke 0,5 aufgezogen. Der Pipetteninhalt wurde bis zur Marke 101 mit Thrombozytenzählflüssigkeit zusammengesetzt aus

Procain-Nilblausulfat	
Procain hydrochlorid	0,4
Nilblausulfat	0,1
NaCl	0,1
aqua dest.	ad 100 ml, ergänzt

Höhe bei einer Verdünnung von 1 200 vom 1 Tag nach der Transplantation bis zum Exitus mit terminalem hämorrhagischem Aszites gezählt.

Histologische Untersuchungen

Leber Milz Niere Lunge und Knochenmark (Sternum) wurden untersucht.

Bakteriologische Untersuchungen

Stichprobenartig wurden Blutkulturen nach Herzpunktion angelegt.

Ehrlich Aszites Karzinom

Die Thrombozytenwerte wurden bei 10 BALB/c Mäusen nach Transplantation von je 1 ml Aszites (= 1 Mio Tumorzellen) eines Ehrlich Aszites Karzinoms bestimmt.

Resultate

Kontrolltiere

Die durchschnittliche Thrombozytenzahl bei Mäusen vom Stamm BALB/c beträgt 975000/mm³ Blut mit einer Standardabweichung von ±175000 (Tab II) Nach BRODSKY [9] ergibt sich bei BALB/c Mäusen ein durchschnittlicher Thrombozytenwert von 983000/mm³ Blut mit einer Standardabweichung von ±183000

Transplantation mit variierender Tumorzellzahl

Gruppen A–F Ein Tag nach der Transplantation wurde bei 72% oder 43 von 60 Mäusen eine Thrombozytopenie mit einem mittleren Wert von 564000/mm³ Blut festgestellt (Tab III) 15 Mäuse oder 25% lagen mit ihren Werten im Bereich der Norm mit einem Mittel von 974000/mm³ Zwei Mäuse zeigten eine Thrombozytose Der Gesamtdurchschnitt der Thrombozytenwerte aller 60 Mäuse belief sich auf 697000/mm³ (Abb 1)

Gruppen G, H und I Zwei Tage nach der Transplantation wiesen 77% der Mäuse oder 23 von 30 eine Thrombozytopenie auf mit einem durchschnittlichen Wert von 608000 Thrombozyten/mm³ Blut (Tab IV) 4 Mäuse zeigten normale Werte, 3 eine Thrombozytose Der Gesamtdurchschnitt aller 30 Mäuse betrug 681000/mm³ (Abb 1)

Gruppe J und K Drei Tage nach der Transplantation wurde bei 75% der Mäuse oder 15 von 20 eine Thrombozytopenie mit einem mittleren Wert von 661000 Thrombozyten/mm³ Blut festgestellt (Tab IV) 5 Mäuse lagen mit ihren Werten im Normbereich mit

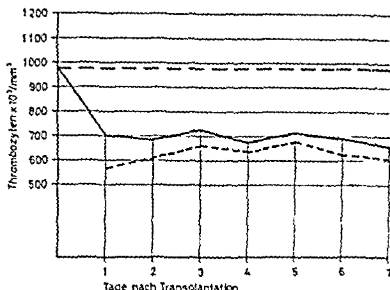


Abb 1 Thrombozytenwerte von 194 BALB c Mäusen Nach i p Inokulation von 1 ml Aszites zu verschiedenen Zeitabschnitten – – Durchschnitt Kontrolltiere, — Gesamtdurchschnitt aller Versuchstiere, --- Durchschnitt der Tiere mit Thrombopenie

Tabelle II Thrombozytenwerte ($\times 10^3/mm^3$) von 30 gesunden BALB c Mäusen = Kontrolltiere

980	930	960
990	1150	990
890	970	980
1100	940	960
910	930	990
940	1030	800
1010	1060	960
870	970	1040
1060	960	970
840	1090	990

einem Mittel von 906000/mm³ Der Gesamtdurchschnitt aller 20 Mäuse betrug 723000/mm³ (Abb 1)

Gruppen L, M und N Vier Tage nach der Transplantation wurde bei 83% der Mäuse eine Thrombozytopenie mit einem Durchschnitt von 633000 Thrombozyten/mm³ Blut gefunden (Tab V) 4 Mäuse zeigten normale Werte Der Gesamtdurchschnitt aller 21 Mäuse betrug 670000/mm³ (Abb 1)

Gruppe O und P Fünf Tage nach der Transplantation zeigten 85% der Mäuse oder 17 von 20 (höchster Prozentsatz) eine Thrombozytopenie mit einem mittleren Wert von 676000 Thrombozyten/mm³ Blut (Tab V) Drei Tiere zeigten normale Werte Der Gesamtdurchschnitt aller 20 Mäuse betrug 711000/mm³ (Abb 1)

Gruppe Q und R Sechs Tage nach der Transplantation wurde bei 80% der Mäuse eine Thrombozytopenie mit einem mittleren Wert von 620000/mm³ Blut gefunden (Tab V) 4 Mäuse zeigten normale Werte (Durchschnitt 645000) Der Gesamtdurchschnitt aller 20 Mäuse betrug 685000/mm³ (Abb 1)

Gruppe S und T Sieben Tage nach der Transplantation zeigten 80% der Mäuse eine Thrombozytopenie mit einem mittleren Wert von 604000 Thrombozyten/mm³ Blut (Tab VI) 4 Mäuse zeigten normale Werte Der Gesamtdurchschnitt aller 20 Mäuse betrug 655000/mm³ (Abb 1)

Histologische Untersuchungen

An Leber Milz, Niere Lunge und Knochenmark der oben erwähnten Gruppen von Mäusen ergaben sich keine pathologischen Befunde, insbesondere kein Plasmozytombefall

Bakteriologische Untersuchungen

Alle von den Versuchstieren angelegten Blutkulturen waren negativ

Ehrlich Aszites Karzinom

Sämtliche Versuchstiere zeigten *normale* Thrombozytenwerte mit einem Gesamtdurchschnitt von 960000/mm³ Blut

Diskussion

Das quantitative Verhalten der Thrombozytenwerte im peripheren Blut bei einem transplantablen Plasmozytom (HIPA Tumor) der BALB/c Maus wurde vom 1 Tag nach der Transplantation bis zum Exitus mit terminalem hämorrhagischem Ascites untersucht. 1–7 Tage nach der i p Inokulation von je 1 ml unverdünnter Aszitesflüssigkeit war bei 80% der BALB/c Mäuse eine Thrombozytopenie mit einem durchschnittlichen Wert von 600000 Thrombozyten/mm³ Blut

Tabelle IV: Thrombozytenwerte von BALB c Mäusen nach i.p. Inokulation von 1 m[l] Aszites (variierende Zellzahl)

Transplantierte Zellzahl $\times 10^4$/ml	Anzahl Empfängertiere	Thrombozyten-Kontroll-Tag	Thrombozytenwerte $\times 10^3$/mm^3
1,45	10	2	1270
			310
			580
			390
			770
			1360
			510
			1020
			700
			590
13,5	10	2	410
			320
			930
			690
			1110
			670
			680
			520
			660
			790
1,90	10	2	750
			680
			650
			810
			590
			630
			660
			670
			940
			740
	10	3	580
			690
			850
			630
			610
			1090
			590
			640
			670
			680
			680
			670

Tabelle IV (Fortsetzung)

Transplantierte Zellzahl $\times 10^6$/ml	Anzahl Empfängertiere	Thrombozyten Kontroll Tag	Thrombozytenwerte $\times 10^3$/mm^3
			810
			590
0 47	10	3	950
			680
			740
			780
			690
			830

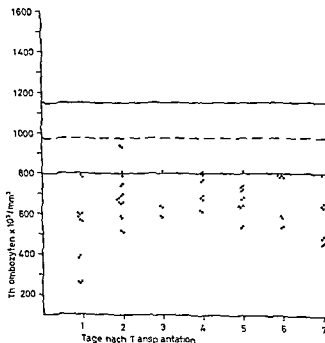


Abb 2 Thrombozytenwerte von 194 BALB/c Mäusen. Nach ı p Inokulanon von 1 ml Asz tes (varuerende Zellzahl) zu verschiedenen Ze tabschn tten

Tabelle V. Thrombozytenwerte von BALB/c Mäusen nach i.p. Inokulation von
Aszites (variierende Zellzahl)

Transplantierte Zellzahl $\times 10^6$/ml	Anzahl Empfängertiere	Thrombozyten-Kontroll-Tag	Thrombozytenw $\times 10^3$/mm³
1,45	5	4	790
			810
			850
			800
			780
7,0	10	4	760
			590
			710
			690
			570
			610
			680
			940
			320
			390
9,6	9	4	320
			520
			830
			540
			740
			620
			780
			670
			770
0,5	10	5	780
			960
			760
			740
			530
			540
			690
			630
			590
			640
	10	5	720
			810
			670
			940
			680
			640
			670
			710

Tabelle 1 (Fortsetzung)

Transplantierte Zellzahl $\times 10^6$/ml	Anzahl Empfängertiere	Thrombozyten Kontroll Tag	Thrombozytenwerte $\times 10^3$/mm³
			780
			730
			780
			530
			590
			580
1	10	6	1120
			830
			790
			980
			430
			590

tion von Thrombozyten in der Peripherie konnen verschiedene Mechanismen in Betracht gezogen werden direkte Viruswirkung [5, 7, 12], immunpathologische Vorgange [7, 19, 27], bakterielle Endotoxine [19]

Onkogene Viren konnten im HIPA Tumorgewebe in verschiedenen Entwicklungsstadien nachgewiesen werden [15] Die reiferen Formen waren den Viren vom Typus C sehr ahnlich, die für mehrere experimentelle Mausleukosen und die sie begleitende Thrombozytopenie verantwortlich sind [7] Bisher konnten beim HIPA Tumor im Gegensatz zu den Mausleukosen keine Viren in den Thrombozyten und Megakaryozyten gefunden werden Unsere Betrachtungen lassen deshalb die Frage offen, ob eine direkte Relation zwischen Viren und Thrombozytopenie besteht

Der sofortige Abfall der Thrombozytenwerte innerhalb 24 h nach der Transplantation spricht gegen immunpathologische Vorgänge als Ursache der Thrombozytopenie Sowohl beim anaphylaktischen Schock wie auch beim generalisierten Sanarelli Shwartzman Phänomen ist das Auftreten von zahlreichen Mikro-Thromben in Lunge und Leber charakteristisch Solche Mikro-Thromben konnten indessen auch nicht beobachtet werden

Intravenos injizierte Bakterien wie z B *Staphylococcus aureus* [19] konnen eine Thrombozytopenie auslosen Die zur Kontrolle angelegten

Tabelle 11 Thrombozytenwerte von BALB/c Mäusen nach i.p. Inokulation von 1 ml Aszites (variierende Zellzahl)

Transplantierte Zellzahl × 10⁶/ml	Anzahl Empfängertiere	Thrombozyten Kontroll Tag	Thrombozytenwerte × 10³/mm³
8 80	10	6	730
			450
			790
			490
			540
			610
			510
			850
			780
			730
8 0	10	7	460
			390
			540
			870
			490
			760
			450
			840
			5[illegible]0
			480
0 99	10	7	780
			790
			760
			650
			680
			810
			590
			630
			640
			920

Blutkulturen waren jedoch alle negativ, so dass eine bakterielle Infektion als Ursache ebenfalls ausscheidet.

In gleichartigen Versuchen mit Tumorzellen eines Ehrlich-Aszites-Karzinoms konnte keine Thrombozytopenie festgestellt werden. Nach den bisherigen Beobachtungen scheint die Thrombozytopenie für das HIPA Plasmozytom pathognomonisch zu sein.

Eine endgültige Erklärung zu geben, weshalb bereits 24 h nach i.p. Inokulation von 1 ml Aszitesflüssigkeit eine Thrombozytopenie mit

einem durchschnittlichen Wert von 600000 Thrombozyten/mm³ Blut auftritt, die vom 1 Tag nach der Transplantation bis zum Exitus konstant bleibt, ist zur Zeit nicht möglich Tatsache ist, dass bei 80 % der Versuchstiere eine statistisch signifikante Thrombozytopenie festgestellt wurde, die unabhängig von der transplantierten Zellzahl ist Das thrombozytopenische Agens scheint keine an Zellen gebundene Substanz zu sein sondern extrazellulär im Exsudat zu liegen

Was den hämorrhagischen Aszites beim HIPA Tumor anbelangt, ist die Thrombozytopenie nicht in der Lage, eine Erklärung für dessen Genese zu geben Zeichen einer allgemeinen hämorrhagischen thrombozytopenischen Diathese fehlen Der hämorrhagische Aszites tritt auch bei den 20 % der tumortragenden Mäuse *ohne* Thrombozytopenie auf Zudem tritt der hämorrhagische Aszites praeterminal auf, die Thrombozytopenie sehr kurze Zeit nach der Transplantation Wir sind deshalb der Auffassung, dass hämorrhagischer Aszites und Thrombozytopenie in keiner direkten Beziehung zueinander stehen und dass die lokal peritoneale Blutung auf eine infiltrativ destruktive Wirkung des Plasmozytoms mit Gefässzerstörung zurückzuführen ist

Zusammenfassung

Das transplantable HIPA Plasmozytom der BALB c Maus ist regelmässig von einem hämorrhagischen Aszites begleitet Es wurde deshalb das quantitative Verhalten der

bedingt
pherie E
Aszites b
rung durch

Summary

The transplantable HIPA plasmocytoma of BALB/c mice is usually associated with hemorrhagic ascites Therefore serial thrombocyte counts were made from the day of transp

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F. G. J. Hayhoe and R. J. Flemans: **An Atlas of Haematological Cytology.**

Man kann sich fragen, ob es heute noch gerechtfertigt sei, einen Atlas von Blut- und Knochenmarksbildern herauszugeben. Wer sich intensiver mit klinischer Hämatologie abgibt, weiss, dass die Morphologie auch in Zukunft einer der wichtigsten Pfeiler der Diagnostik bleiben wird. Die Autoren geben im Vorwort die Richtlinien an, nach welchen sie den Atlas konzipiert haben. Es soll nicht ein Textbuch, sondern eine Referenzsammlung guter Mikroaufnahmen sein, billig, doch von hoher Qualität und in handlichem Format; die Sammlung soll auch in bezug auf Variabilität der Färbungen und Berücksichtigung der Artefakte so realistisch wie möglich sein, es sollen nicht nur speziell photogene Ausschnitte zur Abbildung gelangen. Bei der Durchsicht des kleinen Buches findet man die Absichten der Autoren weitgehend verwirklicht. Etwa 350 Farbaufnahmen sind in vier Kapitel unterteilt: Rote Zellen mit Vorstufen, übrige aus dem Knochenmark stammende Blutzellen mit Vorstufen, Lymphozyten, Plasmazellen und ihre Vorstufen, übrige Zellen aus Blut und Knochenmark, Parasiten.

Die Qualität der Aufnahmen, oder vielmehr ihrer Wiedergabe, vermag nicht immer allen Ansprüchen zu genügen. Viele Bilder erscheinen unscharf oder in der Farbwiedergabe verzerrt. Dank der guten Auswahl der abgebildeten Zellen und der zum Teil sehr starken Vergrösserung kommt aber das Typische dennoch meist gut zur Darstellung. Als ganz besonders wertvoll erweist sich die Abbildung zahlreicher Varianten des gleichen Zelltyps (z. B. rote Vorstufen, Megaloblasten, Lymphoblasten usw.), ferner die vollständige Integration zytochemischer Reaktionen. Dabei beschränken sich die Autoren richtigerweise auf die nicht zuletzt dank den Arbeiten von Hayhoe heute zur Routine gehörenden Färbungen. Es dürfte sich um einen der ersten Atlanten handeln, der das Verhalten der normalen und pathologischen Blut- und Knochenmarkzellen in den verschiedenen Färbungen (v. a. PAS-, Sudan Schwarz-, Peroxydase-, Phosphatasen- und Berlinerblau-Färbung) darstellt.

Die Kapitel werden durch kurze Texte eingeleitet. Die Legenden zu den Abbildungen sind äusserst knapp gefasst. Vom didaktischen Standpunkt aus wäre die Hervorhebung gewisser Zellmerkmale auch in Worten wünschenswert (z. B. generelle Charakteristika der Blasten). Das Buch setzt die Kenntnis der verschiedenen Zelltypen voraus. Die bei zwei oder drei Abbildungen eingestreuten Fragen sind kaum von Nutzen, da die richtigen *Antworten fehlen. Solche Fragen gehören in einen programmierten Text.*

Der kleine, preiswerte Atlas, der praktisch alle wichtigen hämatologischen Bilder einschliesst, wird von allen Laborantinnen und Ärzten, die sich mit morphologischer Hämatologie abgeben, in Ergänzung zur eigenen Präparatesammlung mit grossem Nutzen gebraucht werden. Dem Studenten gibt er die Möglichkeit, einen Überblick über die verschiedenen Blut- und Knochenmarksbilder zu gewinnen.

U. Bucher, *Bern*

Acta haemat 44 321–326 (1970)

Studies on Erythropoiesis Inhibiting Factor (EIF)

II. The site of formation of EIF

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(Director Prof Yasutami Kinoshita)

Recently, it was reported that an erythropoiesistic inhibiting factor (EIF) existed in the plasma of hypertransfused animals [1–3], and in normal rat kidney homogenate [4, 5] Our previous studies also demonstrated that inhibitory activity exists in the plasma from patients with chronic renal failure [6] and with polycythemia vera [7] These findings suggest the possibility that the normal regulation of erythropoiesis is determined by the summation effects of a stimulator, erythropoietin (ESF), and an inhibitor (EIF) However, the site and the mechanism of formation of EIF are not fully understood

In the present study, it was attempted to determine the site of formation of EIF as follows (1) to elevate plasma EIF level, experimental glomerulonephritis was induced in rabbits by a single injection of nephrotoxic serum, and (2) EIF level in the plasma and kidney homogenate from nephritic rabbits was measured before and after bilateral nephrectomy

Materials and Methods

Eight healthy adult male and female rabbits weighing 3 0 to 3 3 kg were used. These rabbits were given i.v a single injection of nephrotoxic serum (0 8 ml/kg) in order to produce glomerulonephritis

Nephrotoxic serum was prepared according to the method of Kozima *et al* [16] as follows ducks were first given 4 i.m or s c. injections of 1 ml sonified rabbit glomeruli in complete Freund s adjuvant (approximately the equivalent of 2 500 glomeruli treated for 5 min with Branson Sonifier S-75 and 0 05 mg killed lyophilized M. tuberculosis H 37 RV/injection) The injections were done at 2 week intervals After this period the

immunization was continued for 5 weeks, twice weekly, with i.p. injections of about 10 000 sonified glomeruli without adjuvant. Eight days after the last injection the ducks were exsanguinated. The individual sera were heated at 56°C for 30 min, absorbed with equal volumes of washed packed rabbit erythrocytes overnight at 4°C and stored until use at −20°C without the addition of preservatives.

Four nephritic rabbits with severe proteinuria (6–8‰) were selected in this study. One week after a single injection of nephrotoxic serum, bilateral nephrectomy was performed under general anesthesia. Then EIF level in plasma and kidney homogenate from nephritic rabbits was measured before and after bilateral nephrectomy.

Whole kidney homogenate was prepared by the homogenizer at 4°C with the free volume of saline. The suspension containing 400 mg of renal tissue (wet weight) per ml was centrifugated, and the supernatant was diluted to the proper concentration before injection into the assay mice.

The inhibitory activity of the plasma or kidney homogenate was tested as follows: 5 ml of the plasma and of the suspension of kidney homogenate from the treated rabbits were mixed with 1.0 U of ESF in 0.2 ml of saline. The mixture was incubated at room temperature for 40 min. One ml (0.2 unit ESF/ml) of the incubated mixture was given s.c. to each of 5 polycythemic assay mice as described by DeGowin et al. [8]. Twenty-four hours after the injection of this mixture each mouse was i.p. injected with 0.5 μCi of ^{59}Fe citrate, and ^{59}Fe incorporation in erythrocytes was determined 2 days later. Blood volume of polycythemic mice was estimated to be 7% of body weight; mice with hematocrits of less than 60% on the day of sacrifice were discarded from the assay. Then, the erythropoietic activity of the mixture was compared with that of controls of ESF (0.2 U) only.

To express the inhibitory activity of EIF quantitatively, we used 'percentage inhibition' as an indicator of the inhibitory effect of EIF in plasma on the known amount of ESF. Percent inhibition was calculated as follows:

$$\left(1 - \frac{\text{Erythropoietic activity of plasma [1 ml]} + \text{ESF [0.2 U]}}{\text{Erythropoietic activity of ESF [0.2 U] only}}\right) \times 100 = \%\ \text{inhibition}$$

The erythropoietin used in this study was extracted from the urine of an anemic patient with bone marrow erythroid hypoplasia according to the method of Lowy and Keighley [9]. The potency of the above urinary erythropoietin was standardized in our assay by comparison with International Standard B Erythropoietin obtained from the Bureau of Standards, National Institute of Medicine, London, England.

Results

Erythropoiesistic inhibiting factor (EIF) in plasma (fig. 1). A single injection of nephrotoxic serum produced glomerulonephritis accompanied by severe proteinuria in rabbits. One week after the injection, slight anemia, and azotemia were noted. The plasma from the rabbits with nephrotoxic nephritis inhibited the erythropoietic activity of ESF. Percent inhibition in 4 nephritic rabbits was on the average 38 ± 12%, while the normal rabbits plasma accentuated the erythropoietic effect of ESF on radioactive iron incorporation in polycythemic mice.

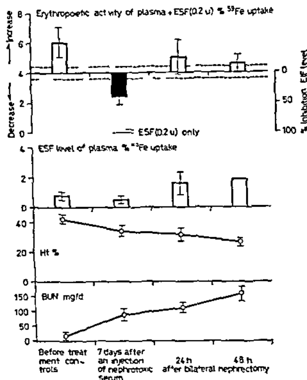


Fig 1 Alterations of erythropoietic activities in 4 rabbits after a single injection of nephrotoxic serum and bilateral nephrectomy Nephritic rabbit plasma after an injection of nephrotoxic serum shows a significant inhibitory effect of ESF (0.2 U) on ^{59}Fe incorporation in polycythemic mice % Inhibition (EIF level) see text.

Saline controls	0.31 ± 0.12	% ^{59}Fe uptake in RBC of 5 polycythemic mice, mean $\pm$ SE
ESF (0.2 U)	4.02 ± 0.37	

On the other hand, the inhibitory activity seen in nephritic plasma rapidly disappeared after bilateral nephrectomy, and, in spite of the elevation of BUN level, the erythropoietic activity in the plasma of nephrectomized rabbits became detected

Erythropoiesistic inhibiting factor (EIF) in kidney homogenate (fig 2) The kidney homogenate from the rabbits with nephrotoxic nephritis had a marked inhibitory action on the effects of ESF in polycythemic mice more than that of normal rabbits It is of interest to note that 200 mg of the nephritic kidney homogenate showed 82% inhibition

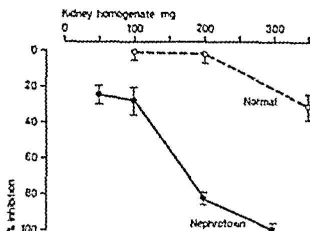


Fig 2 Inhibitory effects of nephrotoxic and normal rabbit kidney homogenates on the erythropoietic effects (^{59}Fe incorporation in RBC) of human urinary erythropoietin (0.2 U) in polycythemic mice

of the effects of 0 2 U ESF, while 350 mg of the normal kidney homogenate showed only 31 % inhibition of ESF In addition, 200 mg of the normal kidney did not produce a detectable inhibition of ESF

Discussion

Since BIRKHILL *et al* [10] have shown that transfusion induced plethora suppresses red blood cell production in man, it is suggested that an erythropoiesistic inhibiting factor (EIF) exists in the plasma of hypertransfused animals [1–3] and in kidney homogenate [4, 5, 11] We demonstrated previously that EIF exists in the plasma from patients with chronic renal failure [6, 15] and with polycythemia vera [7], and attempted to clarify the mechanism of inhibition to ESF by uremic plasma [6, 12]

The findings may suggest that the normal regulation of erythropoiesis is determined by the dynamic balance of ESF and EIF The increase of EIF in uremic plasma may play an important role in the mechanism of anemia of chronic renal failure [6, 12, 15] However, the precise site and the mechanism of formation of EIF are not yet clarified

The present study demonstrates that the plasma from the rabbits with nephrotoxic nephritis shows significantly higher EIF titer than

at of normal ones, but after bilateral nephrectomy the inhibitory tivity rapidly disappears, while ESF activity in the plasma from phrectomized rabbits is detected in spite of the elevation of BUN vel In addition, it is found that the kidney homogenate from the bbits given a single injection of nephrotoxic serum contains sigficantly higher concentration of EIF than that of normal rabbits

These data suggest that the kidney plays an important role in the ite of the production or the activation of EIF, as well as of erythrooietin production It is of interest that MIRAND *et al* [13] have showed hat anephric patients responded to the erythropoietic stimulating ffects of androgen more markedly than did intact patients This sugests that EIF from a diseased kidney may prevent the extrarenal site f ESF production from responding to androgen. As ERSLEV and AZAL [14] have recently reported the presence of EIF not only in idney homogenate but also in liver and spleen homogenate, therefore, urther studies on the problem of the site(s) of EIF production are necessary

Summary

The plasma from rabbits with nephrotoxic nephritis showed higher EIF than that om normal ones However this inh b tory activity rap dly disappeared after bilateral ephrectomy while ESF activity in the plasma from nephrectomized rabb ts was detected t sp te of the elevation of BUN

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Leukocyte Alkaline Phosphatase in Iron Deficiency

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In a recent paper on leukocyte alkaline phosphatase (LAP) activity in rickets due to vitamin D deficiency, several clinical conditions in which LAP studies were previously performed were mentioned [1] Among these iron deficiency anemia was not cited

It is well known that alkaline phosphatase is one of the zinc containing enzymes [2] Zinc deficiency has been clearly demonstrated in patients with the syndrome of iron deficiency anemia, hepatosplenomegaly, dwarfism, hypogonadism and geophagia reported from Iran and Egypt [3–6] Recently we reported 29 children with this syndrome [7] which might indicate that it is not rare in this part of the world Since serum alkaline phosphatase was shown to be increased following treatment in patients with this syndrome [4, 5], we became interested in LAP activity of these patients and in cases of simple iron deficiency anemia

Material and Methods

LAP activity was determined from the leukocyte suspension according to the technic of VALENTINE and BECK [8] which was cited in a previous communication [1] A unit of enzyme activity was defined as the amount of enzyme required to catalyze liberation of 1 mg of phosphorus in 1 h under the standard assay conditions

Blood was obtained from 15 children 2 5 to 14 years of age, who had the syndrome of [illegible]

LAP control values were obtained from 23 children 7 to 36 months of age who were seen either in the well-baby clinic or admitted to the hospital for surgical procedures but who had no evidences of rickets or iron deficiency anemia on clinical, laboratory and

radiologic examination [1]. The children in all 3 groups were free of apparent infection. Iron deficiency anemia was confirmed by low serum iron and increased iron binding capacity in patients with hypochromic microcytic anemia.

Results

When LAP activity was calculated per 10^{10} leukocytes, the mean value for the 23 control children was 36.4 ± 3.2 (SD 15.3) U. In the 15 children with the syndrome it was 56.9 ± 12.9 (SD 46.6) U and for the 17 children with simple iron deficiency anemia 50.6 ± 11.0 (SD 45.8) U (fig. 1b).

Since LAP activity is essentially confined to the mature neutrophils, enzyme activities of these 3 groups were calculated per 10^{10} granulocytes. The mean values were 86.3 ± 15.2; 92.3 ± 18.3 and 85.0 ± 16.2 U in the control children and in patients with the syndrome and simple iron deficiency anemia (fig. 1a). The difference between

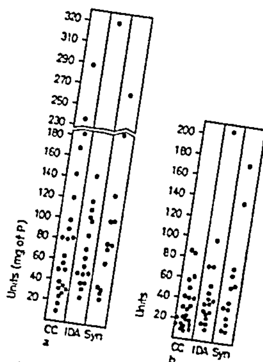


Fig. 1. Leukocyte alkaline phosphatase (LAP) values in control children (CC), in patients with simple iron deficiency anemia (IDA) and with the syndrome of iron deficiency anemia, hepatosplenomegaly, pica, dwarfism and hypogonadism (Syn.). *a* LAP activity per 10^{10} granulocytes. *b* LAP activity per 10^{10} leukocytes.

the values of the control and the simple iron deficiency, between the control and the children with the syndrome and between the simple iron deficiency and the syndrome, was not significant ($p>0.05$), either with values expressed as 10^{10} leukocytes or as 10^{10} neutrophils

Discussion

LAP activity is mainly confined to the mature neutrophils [9, 10], and the ultrastructural localization of this enzyme has also been shown in human neutrophils [11] As it was stated before, alkaline phosphatase is zinc containing enzyme and the zinc content of the myeloid elements increases with the cell maturation [12] Zinc also increases the activity of this enzyme in the leukocytes [13, 14] Although the effect of low serum zinc level on growth retardation has been questioned in patients with the syndrome of iron deficiency anemia, hepatosplenomegaly, pica, dwarfism and hypogonadism, the zinc deficiency in this condition has been well established [3–6, 15] The zinc level of our patients with this syndrome was not studied [7], but it was shown to be decreased in similar cases from this country by ÇAVDAR and ARCASOY [16] Low serum alkaline phosphatase levels have been reported in animals [17] and in patients [5, 15] with zinc deficiency Decreased tissue alkaline phosphatase activity has also been reported in animals with zinc deficiency [18, 19]

But in this study no significant difference was shown between the leukocyte alkaline phosphatase values of the patients with simple iron deficiency anemia and hypogonadism in which zinc deficiency has been shown by several investigators [3–6, 16] Since it is generally stated that in human beings LAP activity bears no relation to serum alkaline phosphatase [20, 21], our results should not be a surprise The rise of serum alkaline phosphatase is a characteristic finding in rickets, but it has been reported that it is not increased in malnourished children with rickets [22] LAP of patients with rickets did not show changes with malnutrition [1], neither is it found in patients with this syndrome in which growth retardation is one of the cardinal findings

Because it is known that LAP remains moderately elevated in infancy and childhood [9, 20], the control values were chosen from children, although their ages were somewhat younger than both study

groups. There was no statistical difference between the control and both the study groups.

Since the leukocyte count is another factor influencing LAP activity [23] the white cell counts in both study groups were compared and not found statistically significant ($p > 0.05$).

From this study it is concluded that LAP activity is not influenced by iron deficiency and/or zinc deficiency.

Summary

Leukocyte alkaline phosphatase (LAP) was determined in 15 children with the syndrome of iron deficiency anemia, hepatosplenomegaly, pica, dwarfism and hypogonadism. Although zinc deficiency and serum alkaline phosphatase changes have been reported in this condition, LAP activity was not different from the cases of simple iron deficiency anemia or the controls. This finding suggests once more that LAP activity is not related to serum alkaline phosphatase changes.

Acknowledgement. For the statistical analysis, I am grateful to Mr. H. R. KIVANÇ and Prof. A. KUTSAL.

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On the Influence of Normal and Leukemic Human Leukocytes on Blood Coagulation[1]

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The blood clot is not only composed of platelets and fibrin network, but also of erythrocytes and leukocytes; in pathologic conditions such as in leukemia, these leukocytes make up a large proportion of the blood and its clots. This study is mainly concerned with the hemostatic properties of the blood leucocyte extracts of healthy subjects, as well as those of patients with chronic granulocytic leukemia, chronic lymphocytic leukemia, acute leukemias, and eosinophilic leukemia. Although a possible role of leukocytes in blood coagulation was pointed out in the last century (BUCHANAN 1845), this problem has only recently attracted attention [10]. These studies have demonstrated that leukocytes may be a source of thromboplastic and antiheparin activities [4, 5, 6, 9, 13, 15, 18, 24, 29], although the relationship between these activities and platelet factors 3 and 4 [14] are still not clear. Thromboplastic activity was also demonstrated in erythrocytes [7, 8].

Material and Methods

Leukocytes were isolated from the blood of 20 healthy donors, 20 patients with chronic lymphocytic leukemia (CLL), 20 patients with chronic granulocytic leukemia (CGL), 6 patients with acute leukemia (AL), and one patient with eosinophilic leukemia (EL). To isolate these leukocytes, the method described by SZMIGIEL [28] was used, to obtain the crude cell extract (called leukocyte extract henceforth), a personal method was used which is described elsewhere [16, 17].

[1] Supported by The Blood Research Foundation, Washington D.C., USA.
Paper delivered at the 13th International Congress of Hematology, Munich, Germany, August 2–8, 1970.

Table I

Leukocytes from	Rec. time	Prothr conspt	ThrombopL gen	Thromb gen	Thromb pl. time
Normal subjects	270	170	30	40	300
Leukemic patients	258	–	66	66	50

Table II

Coagulation tests, mean values	Control, buffer	Leukocytes from				
		Normal subjects	Leukemias			
			CGL	CLL	AL	EL
Plasma recalc. time, sec	126	98	105	87	89	57
Inactivated heparin, µg	–	0.95	0 04	0 03	0 015	0 01
Thrombin time[1]	24	42	45	23	22	23

[1] Thrombin time of substrate plasma (sec) after 30 min incubation of thrombin with cell extracts

The following tests were employed to evaluate the hemostatic activities of these leukocyte extracts *in vitro* recalcification time (rec. time), the prothrombin consumption in the serum [illegible]

[illegible] publications [16, 17] Leukocyte extracts were introduced into the *in vitro* coagulation test system and their effect on the coagulation time of plasma was observed Owren's buffer was added to the control system and observed. Substrate plasma, thrombin solution, heparin solution, and other reagents were prepared according to generally accepted technics [12] In the experiments with extracts of normal leukocytes, various concentrations of these extracts were used (see figures), while in the experiments with leukemic cells the concentration of 1 mg of dry lyophilisates of the cells/1 ml of Owren's buffer was used

Results

The results are summarized in table II and quoted *in figures 1–5* They were submitted to statistical analysis by means of the Student's test, one-way and two-way analysis of variance for equal substrates, and by Ducan's new multiple range test (according to SNEDECOR,

Table III

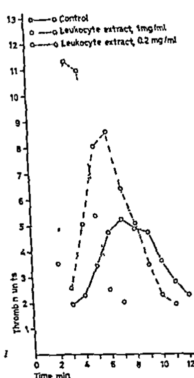
Prothrombin consumption in the serum							
Concentration of leukocyte extract mg/ml	0	0 1	0.2	0 3	0 4	0 5	0 6
Prothrombin time of serum, sec	20	22	24	31	38	47	81

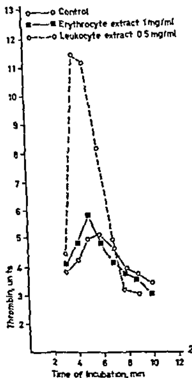
Table IV

Thromboplastin generation in the plasma					
Time of incubation min		2	4	6	8
	platelet extract 1 mg/ml	24	16	12	10
Clotting time of substrate plasma, sec	*leukocyte extract* 1 mg/ml	51	37	28	24
	0 5 mg/ml	52	37	27	23
	0 2 mg/ml	60	42	29	26
	0 1 mg/ml	63	48	37	31

W G Statistical Methods, London 1960, and to WINER, B J: Statistical Principles in Experimental Design, McGraw Hill 1962). From the reported data, it appears that (1) the homogenates of the leukocytes from normal subjects markedly shorten the recalcification time of normal plasma (table II) Furthermore, these leukocyte extracts increase the prothrombin consumption more effectively in the higher than in the lower concentrations (tab III) Besides, it was observed that these extracts can substitute the platelet suspension in the thromboplastin generation test (tab IV), and can induce an increase of the thrombin generation in the plasma (fig 1) The effect of the leukocyte extracts from normal subjects on thrombin generation is higher than the effect of the erythrocyte extracts (fig 2) The extracts of leukocytes from normal persons can significantly shorten the thrombin time of heparinized plasma, but do not influence the thrombin time of normal plasma (fig 3)

(2) The extracts of cells from all leukemic patients showed a statistically significant shortening of the plasma recalcification time





F g 1 Effect of different concentrations of crude normal leukocyte extracts on thromb n generat on in the plasma
F g 2 Comparison of the effects of crude normal leukocyte and erythrocyte extracts on thromb n generat on n the plasma

(tab II) It appeared that all these leukemic leukocyte extracts can substitute the platelet suspension in the thromboplastin generation test (fig 4), and that they increase the thrombin generation in the plasma (fig 5) However, differences were observed in the thromboplastic activities of these various leukemic leukocyte extracts (fig 4 and 5) All cells exhibited an antiheparin activity, since they inactivated heparin in the experimental system 'plasma heparin thrombin buffer' The amounts of heparin inactivated by the cell extracts are presented in table II While the antiheparin activity of leukocytes from normal subjects as well as from both CGL and CLL patients

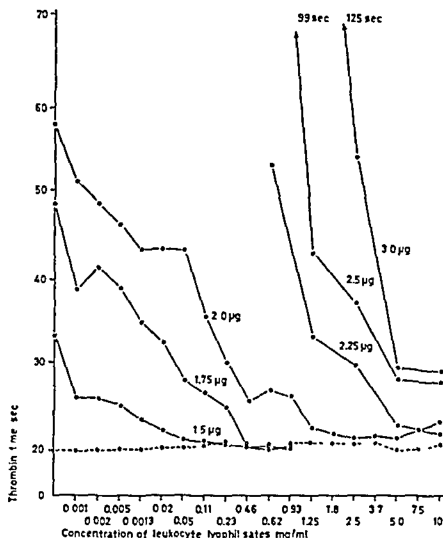


Fig. 3 Effect of crude normal leukocyte extracts on thrombin time of normal plasma (o-- o--- - -o) and heparinized plasma (—o—o—o—)

was relatively high, only a trace of this activity was found in the extract of blast cells from AL and of eosinophils from EL.

An antithrombin effect was demonstrated only in the leukocyte extracts from normal subjects and from patients with CGL (tab. II). It was shown by a progressive decrease in the activity of the thrombin solution, incubated with the above-mentioned leukocyte extracts. In contrast, the extracts of leukocytes of the other leukemias did not exhibit this antithrombin effect.

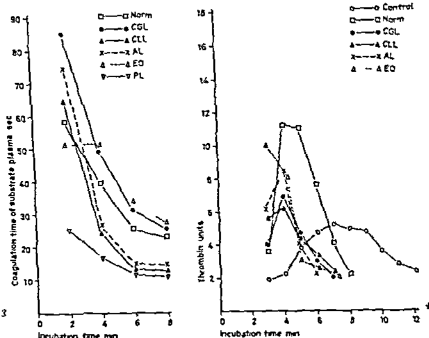


Fig 4 Effect of leukemic leukocyte extracts on thromboplastin generation in the plasma
Fig 5 Effect of leukemic leukocyte extracts on thrombin generation in plasma

Discussion and Conclusions

It was demonstrated that neutrophil leukocytes from normal subjects, invade the thrombi [10] These neutrophils are capable of contributing to the disruption and resolution of the thrombus by mechanical thrombolysis [10, 21] Although the role of lymphocytes and monocytes in the biomorphosis of thrombi are known to a lesser degree, it is possible that at least the monocytes produce the fibroblasts in the thrombus structure [10, 27] Some data indicate, furthermore, the possible influence of the proteolytic enzymes of basophils and of their heparin, histamine and serotonin contents on blood coagulation [8, 25] Only recently, eosinophils [2] have been observed to contain plasminogen [3, 23], and to participate in the resolution of fibrin at inflammation sites [22]

Our study indicates that leukocytes of both normal subjects and leukemic patients exhibit thromboplastic and antiheparin activities. These activities were different in various leukemias. Moreover, the antithrombin activity which is contained in normal leukocytes was also demonstrated in the granulocytes from CGL, but not in the cells from the other leukemias. This substantiates the interpretation that the antithrombin activity is contained only in neutrophils, and not in lymphocytes and blasts, nor in leukemic eosinophils. This is probably due to differences in the enzymatic composition and protein contents [19, 29] of the above-mentioned cells.

The condition of the release of the leukocyte hemostatic activities into the circulating blood is not known. It seems reasonable to suggest that these activities may be manifested also *in vivo* under certain conditions, e.g. after injections of pyrogens, in acute septicemias, in transfusion shock, after transfusion of leukocyte mass, in the Sanarelli-Shwartzman phenomenon [11], and in other thrombohemorrhagic phenomena [26]. Whether the thromboplastic activity of leukocytes may induce intravascular coagulation or thrombosis, remains obscure. Upon autopsy of our patient with EL, a number of heart and vessel thrombi, as well as hemorrhages in the lungs were demonstrated. The possible role of the thromboplastic activity of eosinophils in the above pathological changes is not easy to establish. The exceptionally rare occurrence of eosinophilic leukemias, and their controversial nosologic and pathogenetical patterns [1, 20] make any formation of clinical implications rather difficult.

The nature of the hemostatic activities in the leukocytes from normal beings and leukemic patients is known only poorly. In previous studies [19, 29] it was demonstrated that the antiheparin activity in the granulocytes from CLL is due to the presence of several non-specific basic proteins and enzymes which are not directly involved in the blood coagulation. Probably in other leukocytes this activity is related to various protein components, and not to the specific 'anti-heparin factor'. The general characteristics of the thromboplastic activity of normal leukocytes, discussed in another paper [18], seem to be insufficient for evaluation of the question whether the thromboplastin of various tissues and the thromboplastin of the leukocytes are similar in nature. Thromboplastic material of various leukemic cells has not yet been isolated in purified form. The antithrombin activity of granulocytes, which are rich in lysosomal hydrolytic enzymes,

suggests that its activity is due to proteolytic enzymes in the cell extract. The characterization of the hemostatic activities demonstrated in our investigation with leukocytes from normal subjects and from leukemic patients deserves further investigation.

Summary

Thromboplastic, antiheparin, and antithrombin activity of leukemic leukocytes have been demonstrated. The thromboplastic activity was high in leukemic eosinophils, lymphocytes, and blasts, but lower in mixed normal leukocytes and in leukemic granulocytes. Antiheparin activity was high in mixed normal leukocytes, as well as in leukemic granulocytes and lymphocytes, while leukemic eosinophils and blasts exhibited only traces of this activity. Antithrombin activity was demonstrated in mixed normal leukocytes and in leukemic granulocytes. The possible role of the above activities in the pathogenesis of hemorrhage and in thrombohemorrhagic phenomena is discussed.

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One- and Two Stage Factor VIII-Activity in Acute Leukaemia

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Recently NIEMETZ and NOSSEL [8] made the remarkable observation that in patients with defibrination the factor VIII activity in the plasma was much higher when measured by a 1 stage than when by a 2 stage method This difference was attributed to traces of thrombin present in the plasma in the 1 stage assay activating the factor VIII In the 2 stage method aluminium hydroxide removes the factor VIII activated by thrombin Discrepant 1 and 2 stage factor VIII values in the defibrination syndrome were also noted by MERSKEY *et al* [7]

In the present study the observation of NIEMETZ and NOSSEL have been confirmed and extended and further results of some importance have been obtained

Materials and Methods

Coagulation studies were carried out on blood obtained from (a) 3 patients with acute myeloblastic leukaemia (AML) complicated by defibrination (b) 12 patients with AML without defibrination as assessed by clinical and laboratory evidence and (c) 6 normal controls Blood collected with plastic syringes and 20-gauge needles by clean venepuncture was immediately mixed with 1/10 vol of 4% trisodium citrate dihydrate in a plastic container The plasma was then separated by centrifugation at 2 000 rpm for 15 min at 4 C.

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Hyland AHF standard, the activity of which was frequently compared with fresh plasma whose factor VIII levels were known from comparisons with a pool of plasma samples freshly drawn from 10 normal adults. The results were expressed as percentage of the activity of the Hyland Standard.

The blood euglobulin clot lysis and the euglobulin lysis time were carried out as described by JOHNSON *et al.* [6].

Results

(a) Patients with AML and defibrination. In all 3 patients discrepant 1- and 2-stage factor VIII levels were found. One-stage factor VIII activity was always much higher than the 2-stage one.

(b) Patients with AML without defibrination. In all 12 patients a discrepancy of the 1- and 2-stage factor VIII levels was observed. The discrepancy, however, was not as marked as on patients with defibrination.

(c) Normal subjects. In all 6 normals no discrepancy was found, the correlation between the 2 methods being always good.

Table I. One- and two-stage factor VIII activity in acute myeloblastic leukaemia with and without defibrination

Case No	Sex	Age years	Factor VIII activity %		Platelets mm³	Fibrinogen mg/100 ml	Quick's time, sec
			1 stage	2 stage			
1	M	29	1,750	80	28 000	25	20/12
2	F	18	810	200	15 000	25	26/13
3	M	24	470	80	23 000	30	22/13
4	M	34	222	90	25 000	220	13/12
5	F	17	320	110	18 000	250	14/12
6	F	28	290	100	30 000	320	13/12
7	M	27	282	120	60 000	210	11/11
8	M	19	222	100	15 000	300	12/12
9	M	25	258	105	30 000	400	13/12
10	F	25	320	75	27 000	270	14/12
11	M	40	210	100	70 000	310	13/12
12	M	37	308	100	60 000	300	12/11
13	F	18	290	95	25 000	390	11/11
14	F	24	230	90	15 000	230	13/11
15	M	19	370	105	40 000	270	12/11

Case No. 1–3 with defibrination. Case No. 4–15 without defibrination.

Discussion

In the present study the discrepancy between 1- and 2 stage factor VIII activity observed by NIEMETZ and NOSSEL on patients with defibrination was found, albeit less pronounced, on all patients with acute myeloblastic leukaemia without defibrination One might, perhaps, surmise that on these patients a mild defibrination, undetected by the usual laboratory techniques, takes place During this process minute traces of thrombin are liberated which in turn activate the factor VIII That minute traces of thrombin activate the factor VIII *in vitro* was shown by various authors [2, 8, 9] NIEMETZ and NOSSEL [8] have shown that amounts of thrombin as low as 0 01 U/ml of plasma increased the 1 stage factor VIII activity sevenfold A possible contributory mechanism for the reduction in 2 stage factor VIII-activity would be digestion by plasmin, although such evidence is lacking On the other hand, plasmin does not increase 1 stage factor VIII activity [3]

Summary

In all 12 patients with acute myeloblastic leukaemia without any evidence of defibrination the factor VIII activity in the plasma was higher when measured by a 1-stage than when measured by a 2-stage method

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Partial and Severe Peroxidase and Phospholipid Deficiency in Eosinophils

Cytochemical and Genetic Considerations

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Eosinophil anomaly is generally recognized by a negative peroxidase and phospholipid reaction. Occasionally it is accompanied by hypersegmentation of the nucleus and a scarcity of specific granules. A genetic study of two families of Yemenite origin [20, 22, 23] showed the anomaly to be of autosomal recessive character. The distribution of the defect in the population of Israel has been previously reported [12]. It occurs frequently in ethnic groups living in isolation for several generations [7] and has not been found amongst Europeans.

The present report, describing a genetic follow-up of another family, is brought forth for the following reasons: the family does not come from Yemen, but they are Jews from Persia (not Kurdistan), the child was born with hydrocephaly, and the absence of peroxidase and phospholipids is only partial in several members of the (mother's) family. The phenomena described could be explained on the basis of molecular genetics.

Case Report

An 8-month-old child was sent for routine blood tests from the local antenatal clinic. With the enzyme test routinely used in our laboratory a negative eosinophil peroxidase reaction was discovered. The child was born with hydrocephaly. The possibility of some connection between this condition and the eosinophil anomaly had to be investigated. Four other cases of hydrocephaly from birth were tested. It was decided to test the child's family in order to confirm the hereditary character of the defect.

Materials and Methods

Peripheral blood smears of the propositus and 3 generations of his family were stained according to PAPPENHEIM, GRAHAM KNOLL [8], OSGOOD [19] and a modification of UNDRITZ [26] for peroxidase, and, by ELSON'S method [14] for phospholipids. One hundred eosinophils were counted for segmentation using BESSIS [3] principle. Granulation was estimated subjectively.

Combined test. We wanted to examine whether plasma of the defect carrier contained some factor which can influence the appearance of the defect, and on the other hand whether plasma from a healthy person can cause the disappearance of the defect.

Equal quantities of blood were taken in heparin from both the defect-carrier and a healthy person with the same blood group. It was centrifuged at 3 500 rpm for 10 min. The plasma was removed and exchanged with the other sample. After thorough mixing the samples were incubated for 2 h at 37°C. After centrifugation white corpuscle smears were made and stained as described above.

Results

In order to confirm the hereditary character of the anomaly, the child's family was examined. Eosinophil percentage ranged from 1% in No 15 to 7.6% in No 6. Only very slight hypersegmentation was found in No 8, 17 and 18 and will, therefore, be ignored. Reduced granulation was observed in few eosinophils in No 3, 6, 7, 8, 11, 12, 15 and 18. Negative peroxidase and phospholipid reactions were observed in No 2, 3, 15, 18 and 20, weak peroxidase reactions were found in No 6, 7, 8, 11 and 12.

Figure 1 illustrates the pedigree from which the close relationship between families A and B and the propositus' parents may be seen: mothers of No 1 and 2 are cousins, father of No 3 and mother of No 4 are siblings. Furthermore, No 2's mother is also a sibling in the same family.

The 4 children suffering from hydrocephaly which were tested, showed normal eosinophil peroxidase and phospholipid reactions.

Discussion

BENSO [2] reported reduced peroxidase levels in neutrophil granulocytes in cases of hydrocephaly. No mention is made of peroxidase activity in eosinophils and we are assuming the reaction to have been normal, i.e. positive. The 4 cases of hydrocephaly tested by us showed

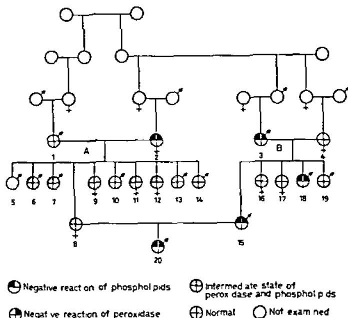


Fig 1 Family tree Partial reaction to peroxidase and phospholipids as found in No 6 7, 8 11 and 12 of the case family

normal eosinophil peroxidase reactions We, therefore, conclude that the 2 phenomena of hydrocephaly and eosinophil anomaly occurred in one patient by coincidence This may arise from segregation of many recessive characters due to the frequent intermarriage of close relatives in the family described A further expression of this may also be the fact that No 17 and 18, siblings of the propositus father, are in institutions for mentally retarded children Negative peroxidase reaction was only found in the uncle (No 18)

LILIE and BURTNER [15] state that there is no connection between peroxidase and phospholipids, however, in all cases we have previously described [20, 23] as well as the present, the parallelism between the reaction of the 2 substances is very evident Similar parallelism is found in polynuclear neutrophils in the Alius Grignaschi anomaly [25]

In a previous paper [21] we have suggested that peroxidase and the phospholipid form one compound The opinion of HALROWITZ [10]

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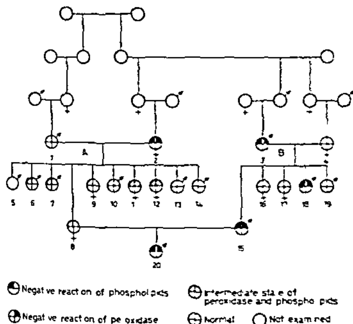


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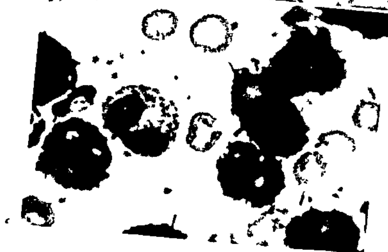
10]



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b



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is both interesting and important in this connection He states that 'in addition to proteins, lipids and especially phospholipids seem to be involved in the formation of electron transferring particles These are disintegrated by bile salts which form complexes with the lipids It is imaginable that the electron transport system is enclosed in an insulating layer of lipoproteins which protects it against the action of proteolytic enzymes or other disintegrating agents, and, also prevents *short-circuits* between neighbouring systems The high lipid content may also be favourable to the penetration of O_2 molecules into the system, since the solubility of oxygen in organic solvents is much higher than that in aqueous systems'

MILLER *et al* [17] concluded that the crystalline substance contains the peroxidase Our findings contradict such a possibility since the granule medulla contains basic proteins and is rich in arginine and it is these substances which give the characteristic staining with eosin and other acid dyes In a previous paper [21] we have shown that the anomaly carrying eosinophils react like normal eosinophils when tested for basic proteins or by the original Sakaguchi method for arginine KELENYI *et al* [13], by combining electron microscopy with peroxidase activity tests, proved that the enzyme is situated in the cortex, i e the outer part of the granules It seems to us that the problem of the position of the enzyme has been solved and since it is situated in the granule cortex, it has no connection with the crystalline substance which is the basic protein

In our previous work [22, 23] we reached the conclusion that the defect is an autosomal recessive character In most cases, enzyme activity is considered a dominant character [6] and therefore no differences are found in heterozygous types The defective phenotype is only found in the homozygote when both alleles lack the activity Two recent theories, those of LYON [16], and JACOB and MONOD [11, 16] can explain the intermediate states which classic genetics could not explain GRUNEBERG [9] though not denying LYON's hypothesis basically, shows that it alone cannot explain the observations which have accumulated, BEUTLER [4, 5], however, explains

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Fig 2 Staining for peroxidase ... neutrophils and eosinophi... partial reaction to perox... case family



with the aid of this theory the diminution in enzyme activity or the activity of autosomal enzymes

We have shown a diminution of peroxidase activity and phospholipid reaction in family A on the propositus mother's side Assuming these people to be heterozygous with only one active allele and wanting to explain the observations on the basis of inactivation of one of the autosomes, we should have found 2 populations The 1st would be with inactivated cells in which the allele is inactive, i e the cells give a positive reaction, while the 2nd, in which no inactivation has occurred, the allele is active and the cells are therefore negative in reaction Since we see no such picture, a similar explanation does not seem probable

We surmise that the anomaly is due to a mutation of the operative gene Grandmother No 2 is homozygous with a pair of inactive alleles, her husband (No 1) is heterozygous with one active and one mutant, partially active allele All the children which inherited the father's active allele are of the normal phenotype, though they are heterozygous Those children which inherited the defective allele are phenotypes with reduced activity of the enzyme It can also be claimed that all No 1 and 2's children are heterozygous and in some of them, No 6, 7, 8, 11 and 12, the defect is only partially expressed But in our opinion the former possibility is more plausible We cannot determine whether using Jacob and Monod's terms [11, 18], a repressor is formed, or, the inducer is formed in insufficient quantity The operator, however, is either not sufficiently active or not at all active We find something similar with thalasemia in which different degrees of haemoglobin F content are found in heterozygotes [1]

Though basically correct, the principle – one *gene*, one *enzyme* – has to be viewed in a new light On the basis of Jacob and Monod's theory [11, 18] the defect in the operon – whether the *structural* or the *operator gene* – has to be determined in every case dealing with a particular enzyme A combination of both Lyon's and Jacob and Monod's theories [11, 16, 18] can probably explain all the cases which Gruneberg showed to be incompatible with Lyon's hypothesis Our

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Fig 3 Staining [illegible]
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observations show that various degrees of enzyme activity are found amongst people carrying the defect This may be logically explained by the influence of the regulating mechanism

Tests of bone marrow taken from a child carrying the defect confirmed that the defect is of genetic origin In all test methods used, the youngest eosinophils bearing characteristic granulation gave negative reactions This was even true of eosinophils during mitosis This fact strengthens our opinion that eosinophils and polynuclear netrophils have no common origin and each line develops separately This phenomenon may be successfully explained on the basis of molecular genetics Since the conclusions for this theory were drawn from work with bacteria, the present case may contribute to under standing that this theory may also explain processes in other living organisms including human beings

Many are convinced that nothing new can be learnt about the well known morphology of blood corpuscules, in contrast, the study of the ultrastructure of healthy and infected cells has made great strides forward Recently, enzymatic methods have been combined with electronic microscopy Clinical cytochemistry has been rather neglect ed in spite of its future being important

Summary

A family lacking eosinophil peroxidase and phospholip ds is described In some members of the family the activity of the enzyme and the phospholip d content are reduced The reason for this is probably a mutation in the regulatory mechanism of the cell. The connec tion between perox dase and phospholip ds requires explanation We confirm our *opinion* that the origin of eosinophils and polynuclear neutrophils is not common. If it were the *same gene* would pass identical information to both types of cell, a thing not observed in the eosinophil or the Alius-Grignaschi anomal es.

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Ers [illegible] the Kaplan Hospital for their assistance in the various stages of the research.

Fig 4 Staining for phospholip d with Sudan Black B Lison's *method* *a* normal bone marrow *b* bone marrow from anomaly carrier the eosinophil myelocyte reacting negatively is in mitosis *c* peripheral blood concentrate from anomaly carrier showing partial react on to phospholip d

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The Distribution of Erythropoietic Bone Marrow in the Mouse

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Experimental work in haematology requires often that certain quantitative data obtained on bone marrow cell suspensions be expressed on a 'per animal' rather than a 'per cell basis Also, in order to obtain a comprehensive picture of experimentally induced changes in some haemopoietic districts, it appears useful to evaluate the amount of marrow in these districts with respect to the total bone marrow of the animal Such estimates require a knowledge of the total volume of bone marrow and of the number of marrow cells in the animal and of their distribution over the entire skeleton

Relevant data obtained by morphological and weight analysis have long been available to this purpose in the rabbit [1, 2] the guinea pig [3], the rat [4, 5], the dog [6] and man [7] More recently, radioisotope tracing techniques have been employed to this end in rats, rabbit, monkeys and men [8, 9, 10, 11]

It has not been possible to find comprehensive data referring to the mouse, a species that has been widely and increasingly used for haematological work in recent years The present paper reports data on the number and distribution of erythroid marrow in the mouse, obtained by standard labelling techniques with ^{59}Fe, that may be of some value for purpose of reference and comparison

Methods

Three month-old m[illegible]
±0 05 g) were injected [illegible]
specific activity 3 mci/r[illegible]

5 animals were killed by exsanguination under ether anesthesia. Blood was collected from the axillary vessels into heparinized vials, counted *in toto* for ^{59}Fe radioactivity, then centrifuged at 3,000 rpm for 10 min and counted again for the activity in plasma and packed red blood cells.

The carcasses were skinned, dried in the oven at 60°C overnight and then placed separately into colonies of the insect *Dermestes frischii*, which feed on the flesh and leave the bones perfectly clean. The various bones were then dissected, weighed and counted separately in a well type scintillation spectrometer. A standard aliquot of the injected ^{59}Fe solution was also counted with each set of samples, in order to normalize the counts. This experiment was repeated twice, for a total of 120 animals used.

Blood volume was estimated by a standard dilution method with ^{59}Fe-labelled isologous erythrocytes and yielded a normal value of 1.9±0.1 ml on 10 mice in the range of weight between 23 and 25 g.

Estimates of bone marrow cell number were carried out on 10 animals injected with $10\ \mu ci$ of ^{59}Fe and sacrificed 6 h later. The epiphyses of one femur were cut by a circular saw and the femur shaft was flushed repeatedly with Hank's solution. The bone marrow was thoroughly suspended and cells were counted with a Bürker haemocytometer. The remainder of the skeleton was then counted for radioactivity, together with the flushed femur shaft and the epiphyses. The formula used for the calculation of the total bone marrow cells was the following:

$$N = N_f \times \frac{A_{sk}}{A_e},$$

where N = total number of nucleated bone marrow cells in the animal, N_f = number of nucleated cells in the femur shaft, A_{sk} = radioactivity in the whole skeleton, A_e = radioactivity in the flushed bone marrow. A_e was obtained as the difference in activity between the unflushed and the flushed femur.

Results and Discussion

Figure 1 shows the changes in the uptake of ^{59}Fe in the whole blood, packed red cells and the skeleton, as a function of time after the injection of the isotope. Values for the skeleton are expressed in cpm/g of bone, values for the whole blood and the packed erythrocytes in cpm/ml. It is seen that by 6 h after injection the clearance of ^{59}Fe from the plasma is practically complete and the activity in the skeleton reaches its maximum. A definite change in the slope of the curve of blood radioactivity takes place from the first day on, with a shallow rise between day 1 and day 7. In parallel, the loss of ^{59}Fe from the skeleton is marked from 6 to 24 h and much less pronounced from this time on. These data, together with the measurements of total blood volume mentioned previously show that by 24 h the amount of radioactivity released in the blood of a mouse is about 50% of the injected activity.

The percentage of the injected ^{59}Fe found at various times in various bone districts is shown in figure 2. A peak value of uptake is observed

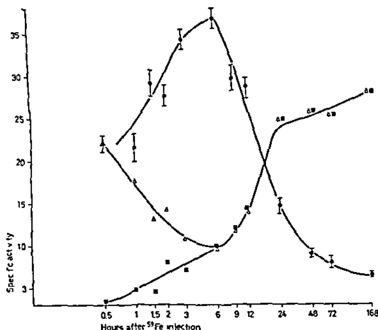


Fig 1 Changes in the uptake of ^{59}Fe in the whole blood (Δ——Δ cpm/ml × 10^{-3}), packed red cells (■——■ cpm/ml × 10^{-3}) and skeleton (●——● cpm/g bone × 10^{-3}) as a function of time after radioisotope injection

Table I Percent contribution of the various mouse bones to the activity of the total skeleton excluding the skull

Vertebral column	42 6±0.5
Pelvis	13 0±0 4
Femur (2)	12 2±0 2
Sternum and ribs	12 1±0 2
Tibia and fibula (2)	6 7±0 1
Humerus and clavicle (2)	6.5±0 2
Scapula (2)	3.2±0 1
Ulna rad us and forefoot (2)	2 2±0 2
H nd foot (2)	1 3±0 3

at 6 h in all districts, followed by a decrease, a pattern similar to that shown in the same figure for the entire skeleton The loss of activity from the skull proceeds at a lower rate, compared to the other bones, and this is due to the presence of ^{59}Fe in tooth matrix, a phenomenon

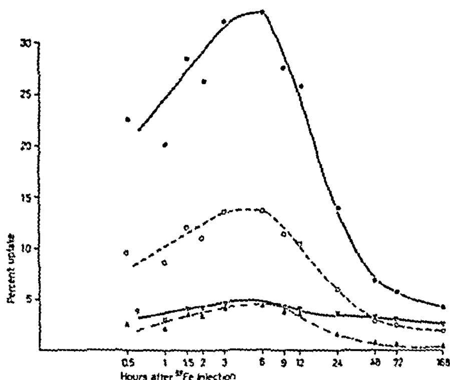


Fig. 2. Variations with time of the percentage of injected ^{59}Fe in various bone districts and in the whole skeleton. ● = Total skeleton (excluding skull), ○ = vertebrae, ▲ = femur, ▽ = skull.

already found by BELCHER *et al.* [12]. The 2nd experiment was carried out to analyze the significance of this finding and the relevant data are shown in figure 3. It is seen here that for the first 9 h after ^{59}Fe injection the pattern of uptake in the teeth with respect to the standard and to the uptake of the skull is similar. However, there is a difference in the pattern at later times when the uptake in the teeth equilibrates with respect to the standard but continues to increase as a percentage of the activity in the skull. Although the weight of the teeth is 30% of the skull, the activity retained at one week is over 60%. If allowance is made for the skull uptake (which is 13% at 6 h, 19% at 24 h and 34% at one week of the activity in the skeleton) the contribution of each bone district to the radioactivity of the whole skeleton appears to be quite constant within our observation time. The percentage of ^{59}Fe in various bones with respect to the radioactivity of the whole skeleton is given in table I. The figures in this table are averages for all mice in the experiments.

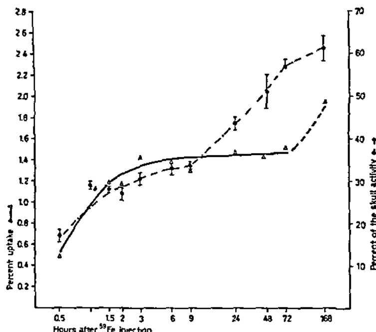


Fig 3 Patterns of ^{59}Fe uptake in the teeth with respect to the standard (left side ordinate) and to the uptake of the skull

The changes with time of the specific activity (cpm/g of bone) of the various parts of the bony skeleton are given in figure 4 Again, the peak is observed at about 6 h and the values pertaining to the bone districts at this time are summarized in table II

In separate experiments attempts were made to evaluate the total number of nucleated haematopoietic bone marrow cells, following the technique described previously Over a total of 10 animals this number was found to be $2\ 4 \pm 0\ 3 \times 10^7$ nucleated cells/g body weight

Under the assumption that the rate of haemoglobin synthesis in erythropoietic cells is the same in all bone districts examined, table I provides an estimate of the amount of erythropoietic marrow in various sections of the skeleton The above assumption is rendered likely by the constance of the percent contribution of the various bone districts to the total activity of the skeleton throughout the observation period of 7 days, with the exception of the skull, as discussed previously

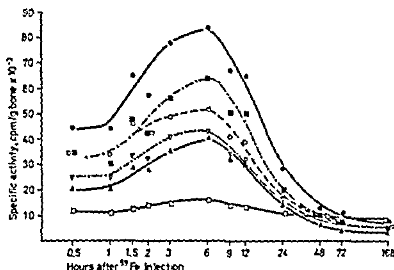


Fig. 4. Variations with time of the specific activity (cpm/g bone × 10^{-3}) in various bone districts. ● = Pelvis, ■ = femur, ▽ = vertebrae, ○ = sternum and ribs, ▲ = tibia and fibula, □ = skull.

Table II. ^{59}Fe specific activity (cpm/g of bone × 10^{-3}) of various mouse bones at 6 h after radio isotope injection

Pelvis	84 ± 3
Femur (2)	64 ± 3
Humerus and clavicle (2)	61 ± 3
Sternum and ribs	52 ± 2
Scapula (2)	44 ± 1
Vertebral column	43 ± 2
Tibia and fibula (2)	40 ± 2
Ulna, radius and forefoot (2)	12 ± 1
Hind foot (2)	2 ± 1

The vertebral column is the compartment with the highest absolute content of red marrow, both in terms of injected radioactivity (fig. 2) and in terms of skeleton radioactivity (table I). The femura, often used in experimental work, contain about 12% of the total marrow.

A different situation is shown in figure 4 and table II where the specific activity would indicate the *density* of the erythropoietic tissue in the various bones. The pelvis and the femura show in this case the highest concentration of ^{59}Fe/g of bone.

In general, the distribution of ^{59}Fe throughout the bone skeleton of the mouse is remarkably similar to that found in the rat by LAMERTON *et al* [8]

The estimate of nucleated marrow cells in a mouse, obtained in the present series of experiments is well within the range of values reported previously for other mammalian species and in very good agreement with other data on the mouse which appeared while the present paper was in preparation [13]

Summary

The distribution of erythropoietic bone marrow in the skeleton of the mouse has been investigated with ^{59}Fe tracing techniques The incorporation of ^{59}Fe reaches its maximum at 6 h after radioisotope injection Evaluation of the total amount of marrow in the skeleton gives $2.4 \pm 0.3 \times 10$ nucleated cells/g body weight. The femura often used in experimental haematology contain 12% of the total marrow and the 2nd highest concentration of erythropoietic tissue/g of bone Differences in the iron uptake to the skull because of the presence of the teeth are also reported

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R Richterich, H Ehrengruber und H R Tschanz Internationale Klassifikation der Krankheiten Datenverarbeitung in der Medizin Monographie, Vol 1 Karger, Basel 1968 VIII + 88 pp , sFr 25 –

Es handelt sich hier um die deutsche Übersetzung der von der WHO 1967 heraus gegebenen 8 Revision der «International Classification of Diseases» Es ist von den Autoren und vom Verlag Karger äusserst verdienstvoll im Zeitalter der hypertrophierenden Amerikanismen eine deutschsprachige Fassung der Krankheitsstatistik geschaffen und damit zur Verbreitung einer einheitlichen Klassierung wesentlich beigetragen zu haben Das übersichtlich angeordnete Verzeichnis mit Griffregister erlaubt eine rasche Orientierung Es umfasst rund 1000 dreistellige Codenummern von denen jede noch durch eine weitere Ziffer in 10 Unterkategorien eingeteilt werden kann Der Diagnosenschlüssel ist computergerecht programmiert Er soll vor allem der Krankenhausstatistik dienen, ist aber mit geringfügigen Modifikationen auch für wissenschaftliche Dokumentation geeignet

H Lüdin, Basel

Handbuch der inneren Medizin Begr v L. Mohr und R Staehelin Vol 2 Blut und Blutkrankheiten, L. Heilmeyer (Hrsg), 5 Aufl 1 Teil Allgemeine Hämatologie und Physiopathologie des erythrozytären Systems Springer, Berlin 1968 XVIII + 786 pp , DM 235 –/US $ 47 –

Während in der 4 Auflage dieses Handbuches (1951) die Hämatologie in einem einzigen Band von 2 Autoren behandelt wurde, haben wir in der nun erscheinenden 5 Auflage den ersten Teil eines vierbändigen Werkes vor uns, an dem über 30 Autoren mitwirken Einmal mehr spiegeln sich hier die gewaltige Entwicklung der Hämatologie und die Vertiefung unserer Kenntnisse im Laufe der vergangenen 15 Jahre wider Dies wird bereits bei Durchsicht dieses 1 Teiles, der ausschliesslich die Grundlagen behandelt, deutlich Es seien vor allem die Kapitel über die Plasmaeiweisskörper, die Zytochemie, die Anwendung von Isotopen und die Biochemie der Erythrozyten und des Hämoglobins genannt, die eine Vielfalt neuester Forschungsergebnisse vermitteln. Das zuletzt genannte Kapitel umfasst auch die Hämoglobinopathien Jeder Abschnitt schliesst mit einem ausführlichen, die neuesten Publikationen berücksichtigenden Literaturverzeichnis Trotz der Vielzahl von Autoren besitzt der Band einen einheitlichen Aufbau Er ist durch zahlreiche instruktive Abbildungen illustriert, wobei die schönen Reproduktionen farbiger Mikrophotographien besondere Beachtung verdienen

H Lüdin, Basel

A M Ganzoni Kinetik und Regulation der Erythrozytenproduktion. Springer, Berlin 1970 VIII + 94 pp , 43 fig , DM 48 –/US $ 10 50

Diese Monographie enthält eine Fülle von experimentellen Daten die der Autor auf grund eigener Untersuchungen am Tiermodell gewonnen hat Die Monographie mag den klinisch orientierten Hämatologen zunächst als hochspezialisierte Arbeit abschrecken Ganzoni versteht es jedoch, den Leser zunehmend an den biologischen Vorgängen der Erythrozyten und Hämoglobinbildung sowie der damit verbundenen Regulationsmechanismen zu interessieren Seine kritischen Interpretationen von Isotopenmethoden die für Untersuchungen des Eisenstoffwechsels häufig angewandt werden sind für die Klinik von grösster Bedeutung Die neuere Literatur über die genannten Probleme wird kritisch gewürdigt Offene Fragen werden klar als solche dargestellt Das Buch darf jedem an Regulationsmechanismen interessierten Hämatologen klinischer oder biochemischer Richtung wärmstens empfohlen werden

E. A Beck, Bern

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